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(54) Title: USE OF A COMPOSITION WHICH REGULATES OXIDATION/REDUCTION REACTIONS INTRACELLULARLY AND/OR EXTRACELLULARLY IN A STAINING OR SORTING PROCESS OF SPERMATOZOA

(57) Abstract: Staining mixtures comprising viable spermatozoa, a composition which regulates oxidation/reduction reactions intracellularly or extracellularly, and a DNA selective dye are disclosed. The cells contained in such suspensions tend to have a greater capacity for enduring the various process steps typically associated with the sorting of sperm cells into gender enriched populations, thereby resulting in post-sort compositions with an increased number of viable or motile sperm. Processes for staining sperm cells comprising the formation of a staining mixture are also disclosed.

WO 2005/095960 A1

USE OF A COMPOSITION WHICH REGULATES OXIDATION/REDUCTION REACTIONS  
INTRACELLULARLY AND/OR EXTRACELLULARLY IN A STAINING OR SORTING PROCESS OF  
SPERMATOZOA

FIELD OF THE INVENTION

5 [0001] The present invention generally relates to a process of sorting stained sperm cells. More specifically, the present invention relates to processes for sorting sperm cells in which a sperm cell suspension containing a composition which regulates oxidation/reduction reactions 10 intracellularly and/or extracellularly is formed.

BACKGROUND

15 [0002] The fertilization of animals by artificial insemination (AI) and embryo transplant following *in vitro* fertilization is an established practice. In the livestock production industry, the ability to influence the reproductive outcome toward offspring having one or more desired characteristics has obvious advantages. By way of example, there would be an economic benefit in the dairy industry to preselect offspring in favor of the female sex 20 to ensure the production of dairy cows. The separation of sperm into enriched populations of X and Y chromosome-bearing cells, known as gender enriched semen or gender enriched sperm, is one method of achieving preselected offspring.

25 [0003] In order to obtain gender enriched semen, sperm cells must be stained with a dye and subsequently sorted into X and Y chromosome-bearing cells. Each of staining and sorting processes places a stress on the sperm cells that decreases sperm cell viability or motility, 30 particularly progressive motility. Especially stressful is the process of staining the sperm cells, which requires contacting the cells with a dye for a certain period of

time, often at a temperature and pH which are not common in the typical sperm cell environment.

SUMMARY OF THE INVENTION

5 [0004] Among the various aspects of the present invention are sperm suspensions having utility, for example, in processes used to sort sperm into enriched populations of X or Y-chromosome bearing sperm.

10 [0005] Briefly, therefore, the present invention is directed to a staining mixture comprising viable spermatozoa, a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly, and a DNA selective dye, the concentration of the composition in the staining mixture being greater than 50 $\mu$ M when the composition is pyruvate.

15 [0006] The present invention is further directed to a process for staining sperm cells, the process comprising forming a staining mixture containing intact viable sperm cells, a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly, and a DNA selective dye, the concentration of the composition in the staining mixture being greater than 50 $\mu$ M when the composition is pyruvate.

25 BRIEF DESCRIPTION OF THE DRAWINGS

[0007] FIGURE 1 graphically depicts the results of the study carried out in Example 1 wherein percent progressive motility of sperm is measured for sperm stained with 400 $\mu$ M Hoechst 33342 dye at 41°C in either a TCA buffer or a TCA buffer containing 10mM pyruvate.

[0008] FIGURE 2 graphically depicts the results of the study carried out in Example 2 wherein percent progressive

motility of sperm is measured for sperm stained with 400 $\mu$ M Hoechst 33342 dye at 41°C in either a TCA buffer or a TCA buffer containing 10 $\mu$ M vitamin K.

[0009] FIGURE 3 graphically depicts the results of the 5 study carried out in Example 3 wherein percent progressive motility of sperm is measured for sperm stained with 400 $\mu$ M Hoechst 33342 dye at 41°C in either a TCA buffer or a TCA buffer containing 100 $\mu$ M vitamin K.

[0010] FIGURE 4 graphically depicts the results of the 10 study carried out in Example 4 wherein percent progressive motility of sperm is measured for sperm stained with 400 $\mu$ M Hoechst 33342 dye at 41°C in either a TCA buffer or a TCA buffer containing 1mM lipoic acid.

[0011] FIGURE 5 graphically depicts the results of the 15 study carried out in Example 5 wherein percent progressive motility of sperm is measured for sperm stained with 600 $\mu$ M Hoechst 33342 dye at 28°C in either a TCA buffer or a TCA buffer containing 10mM pyruvate.

[0012] FIGURE 6 graphically depicts the results of the 20 study carried out in Example 6 wherein percent progressive motility of sperm is measured for sperm stained with 600 $\mu$ M Hoechst 33342 dye at 28°C in either a TCA buffer or a TCA buffer containing 100 $\mu$ M vitamin K.

[0013] FIGURE 7 graphically depicts the results of the 25 study carried out in Example 7 wherein percent progressive motility of sperm is measured for sperm stained with 600 $\mu$ M Hoechst 33342 dye at 28°C in either a TCA buffer or a TCA buffer containing 1mM lipoic acid.

[0014] FIGURE 8 graphically depicts the results of the 30 study carried out in Example 8 wherein percent progressive motility of sperm is measured for sperm stained with 600 $\mu$ M Hoechst 33342 dye at 28°C in a TCA buffer, a TCA buffer containing 2.5mM pyruvate, a TCA buffer containing 10mM

pyruvate, a TCA buffer containing 25mM pyruvate, and a TCA buffer containing 50mM pyruvate.

[0015] FIGURE 9 graphically depicts the results of the study carried out in Example 9 wherein percent progressive motility of sperm is measured for sperm stained with 20 $\mu$ M SYBR-14 dye at 28°C in either a TCA buffer or a TCA buffer containing 10mM pyruvate.

[0016] FIGURE 10 graphically depicts the results of the study carried out in Example 10 wherein percent progressive motility of sperm is measured for sperm stained with 100 $\mu$ M BBC dye at 28°C in either a TCA buffer or a TCA buffer containing 10mM pyruvate.

[0017] FIGURE 11 graphically depicts the results of the study carried out in Example 11 wherein percent progressive motility of sperm is measured for sperm stained with 200 $\mu$ M BBC dye at 28°C in either a TCA buffer or a TCA buffer containing 10mM pyruvate.

[0018] FIGURE 12 graphically depicts the results of the study carried out in Example 12 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 600 $\mu$ M Hoechst 33342 dye at 28°C in TCA containing 10mM pyruvate or in carbon dioxide-blanketed TCA containing 10mM pyruvate.

[0019] FIGURE 13 graphically depicts the results of the study carried out in Example 12 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 600 $\mu$ M Hoechst 33342 dye at 28°C in TCA containing 10mM pyruvate or a carbonate-based inhibitory buffer at pH 7.3.

[0020] FIGURE 14 graphically depicts the results of the study carried out in Example 12 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 600 $\mu$ M Hoechst 33342 dye at 28°C in TCA

containing 10mM pyruvate or a carbonate-based inhibitor at pH 6.2.

[0021] FIGURE 15 graphically depicts the results of the study carried out in Example 13 wherein percent 5 progressive motility of sperm cells is measured for sperm cells stained with 1000 $\mu$ M Hoechst 33342 dye at 28°C in TCA containing 10mM pyruvate and then diluted 1 to 3 with either TCA containing 10mM pyruvate or a carbonate-based inhibitor at pH 6.2.

10 [0022] FIGURE 16 graphically depicts the results of the study carried out in Example 13 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 1000 $\mu$ M Hoechst 33342 dye at 28°C in (1) TCA containing 10mM pyruvate and diluted 1 to 3 with the 15 same or (2) a carbonate-based buffer at pH 7.3 and diluted 1 to 3 with carbonate-based inhibitor at pH 6.2.

[0023] FIGURE 17 graphically depicts the results of the study carried out in Example 13 wherein percent progressive motility of sperm cells is measured for sperm 20 cells stained with 1000 $\mu$ M Hoechst 33342 dye at 28°C in TCA containing 10mM pyruvate or a carbonate-based inhibitor at pH 6.2.

[0024] FIGURE 18 graphically depicts the results of the study carried out in Example 14 wherein percent 25 progressive motility of sperm cells is measured for sperm cells stained with 300 $\mu$ M Hoechst 33342 dye at 41°C in TCA containing 10mM pyruvate and then diluted 1 to 3 with either TCA containing 10mM pyruvate or a carbonate-based inhibitor at pH 6.2.

30 [0025] FIGURE 19 graphically depicts the results of the study carried out in Example 14 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 300 $\mu$ M Hoechst 33342 dye at 41°C in (1)

TCA containing 10mM pyruvate and diluted 1 to 3 with the same or (2) a carbonate-based buffer at pH 7.3 and diluted 1 to 3 with carbonate-based inhibitor at pH 6.2.

[0026] FIGURE 20 graphically depicts the results of 5 the study carried out in Example 14 wherein percent progressive motility of sperm cells is measured for sperm cells stained with 300 $\mu$ M Hoechst 33342 dye at 41°C in TCA containing 10mM pyruvate or a carbonate-based inhibitor at pH 6.2.

10

DETAILED DESCRIPTION OF THE INVENTION

[0027] Surprisingly, it has been determined that 15 spermatozoa contacted with a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly tend to have a greater capacity for enduring the various process steps typically associated with the sorting of sperm cells into an enriched population of X or Y chromosome-bearing spermatozoa. In a preferred embodiment, therefore, gender enriched populations of 20 spermatozoa may be prepared for artificial insemination which have an increased number of viable cells or an increased number of motile sperm, particularly progressively motile sperm, in a post-stain or post-sort composition.

[0028] In general, a composition which regulates 25 oxidation/reduction reactions intracellularly and/or extracellularly is a composition comprising a component that can transfer electrons from one substance to another. Such a composition may comprise a component that either 30 gains or scavenges electrons (an oxidizing agent or electron acceptor) or a component that donates electrons (a reducing agent or electron donor). With respect to

biological systems, a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly is more easily understood to be a composition that either adds or removes an oxygen or 5 hydrogen from a compound.

[0029] Generally, therefore, such a composition may comprise, for example, pyruvate, vitamin K, lipoic acid, glutathione, flavins, quinones, superoxide dismutase (SOD), and SOD mimics. Such a composition may be present in sperm 10 suspension in a concentration sufficient to effect the protective effect without detrimentally affecting sperm health. Exemplary concentration ranges include from about 10 $\mu$ M to about 50mM depending upon such factors as the particular composition being used or the concentration of 15 sperm in the suspension. For example, if pyruvate is included in the composition, it may be present in the sperm suspension in a concentration from about 0.5 $\mu$ M to about 50mM, preferably from about 1mM to about 40mM, more preferably from about 2.5mM to about 25mM, still more 20 preferably from about 10mM to about 20mM, even still more preferably at about 15mM, and most preferably at about 10mM. If vitamin K is included in the composition, it may be present in the sperm suspension in a concentration from about 1 $\mu$ M to about 100 $\mu$ M, preferably from about 10 $\mu$ M to 25 about 100 $\mu$ M, more preferably from about 50 $\mu$ M to about 100 $\mu$ M, and most preferably at about 100 $\mu$ M. If lipoic acid is included in the composition, it may be present in the sperm suspension in a concentration from about 0.1mM to about 1mM, preferably from about 0.5mM to about 1mM, more 30 preferably about 0.5mM, and most preferably about 1mM. The sperm suspension may comprise any one of the above listed embodiments of the composition or any combination thereof

in the above listed concentrations. For example, the sperm suspension may comprise a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly comprising pyruvate in a concentration of 5 about 10mM and vitamin K in a concentration of about 100 $\mu$ M. Alternatively, the sperm suspension may comprise a composition comprising pyruvate in a concentration of about 10mM and lipoic acid in a concentration of about 1mM. Yet another example includes a sperm suspension comprising a 10 composition comprising pyruvate in a concentration of about 10mM, vitamin K in a concentration of about 100 $\mu$ M, and lipoic acid in a concentration of about 1mM.

[0030] Generally, a composition which regulates oxidation/reduction reactions intracellularly and/or 15 extracellularly may provide a protective effect such as an increase in the number of viable cells or an increase in the number of motile cells, particularly progressively motile cells, in the sperm suspension containing the composition. While such a composition provides a 20 protective effect to any suspension formed in the process of sorting sperm cells, the benefit is of particular value during the staining step, wherein such a composition may help to maintain sperm viability at elevated staining temperatures, at elevated dye concentrations, at increased 25 staining periods, or any combination thereof.

[0031] In general, the cell sorting process comprises a series of discrete steps, i.e., collection of a cell sample, staining of the cells, sorting of the cells, collection of the sorted cells, and optionally, 30 cryoextension of the sorted cells. Advantageously, the composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly may be included in

sperm suspensions formed or employed in one or more of these steps.

**Collection of the cell sample**

[0032] Intact viable bovine, porcine, equine, or other 5 mammalian sperm cells, may be collected and contacted with the motility inhibitor. Various methods of collection of viable sperm are known and include, for example, the gloved-hand method, use of an artificial vagina, and electro-ejaculation. As an example, a bovine semen sample, 10 typically containing about 0.5 to about 10 billion sperm cells per milliliter, may be collected directly from the source mammal into a vessel containing a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly to form a sperm suspension.

15 Alternatively, the semen sample may be collected into an empty vessel and then subsequently contacted with such a composition within several hours after collection to form the sperm suspension.

[0033] The sperm sample may also be combined with a 20 buffer (in the form of a solid or solution) to form a buffered sperm suspension. Among other things, the buffer may enhance sperm viability by buffering the suspension against significant changes in pH or osmotic pressure. Generally, a buffer is non-toxic to the cells and is 25 compatible with the dye used to stain the cells. Exemplary buffers include phosphates, diphosphates, citrates, acetates, lactates, and combinations thereof. Presently preferred buffers include TCA, TEST, sodium citrate, HEPES, TL, TES, citric acid monohydrate, HEPEST (Gradipore, St. 30 Louis, MO), PBS (Johnson et al., *Gamete Research*, 17:203-212 (1987)), and Dulbecco's PBS (Invitrogen Corp., Carlsbad, CA).

[0034] One or more buffers may be combined together or with additives as discussed below to form a buffered solution, and the buffered solution combined with the sperm sample to form a buffered sperm suspension. A buffered solution may also contain one or more additives, as described in greater detail below, or a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly. Exemplary buffered solutions are described in Table I. Preferred buffered solutions include

5 a solution comprising 3% TRIS base, 2% citric acid monohydrate, and 1% fructose (w/v) in water at a pH of about 7.0, a solution designated as TCA #1 in Table I, and

10 a solution designated as TCA #2 in Table I.

Table I. Buffered Solutions

COMPONENTS	TCA#1	TCA#2	TEST	Na Citrate	HEPES	TL
Sodium chloride (NaCl)					7.6g	5.84g
Potassium chloride (KCl)					0.3g	0.23g
Sodium bicarbonate (NaHCO <sub>3</sub> )						2.1g
Sodium phosphate monobasic (NaH <sub>2</sub> PO <sub>4</sub> ·H <sub>2</sub> O)						0.04g
(+)-2-hydroxypropionic acid (Na Lactate)						3.68ml
Magnesium chloride (MgCl <sub>2</sub> )					0.1g	0.08g
N-(2-hydroxyethyl)piperazine-N'-(2-ethansulfonic acid) (HEPES)					2.38g	2.38g
tris(hydroxymethyl) amimonethane (TRIS base)	30.3g	32.02g	10.28g			
Citric Acid Monohydrate	15.75g	18.68g				
Na Citrate Dihydrate				29g		
2-[(2-hydroxy-1,1-bis[hydroxymethyl] ethyl)aminoethanesulfonic acid (TES)]			43.25g			
Fructose	12.5g	2.67g		10g	2.52g	
D-Glucose			2g			
Steptamycin			0.25g			
Penicillin-G			0.15g			
Water	1 liter	1 liter	1 liter	1 liter	1 liter	1 liter
Target pH	7.35	7.35	7.35	7.35	7.35	7.35
Target osmolality (milliosmols/kg H <sub>2</sub> O)	-314	-300	-302	-316	-298	-296

[0035] The amount of buffer employed generally depends upon several considerations, e.g., the particular buffer and the desired sperm concentration (# sperm/ml) in the

buffered sperm suspension. Therefore, a sufficient amount of buffer will be used such that the desired concentration of sperm/ml is achieved. Buffer may be added to achieve a sperm suspension that contains from about  $1 \times 10^3$  sperm/ml to about  $5 \times 10^{10}$  sperm/ml. For example, in one embodiment buffer may be added to achieve a "relatively low" concentration of sperm in the sperm suspension, i.e., buffer is added to achieve a sperm suspension that contains less than about  $1 \times 10^7$  sperm/ml, preferably less than about 10  $1 \times 10^6$  sperm/ml, more preferably about  $1 \times 10^3$  to about  $5 \times 10^6$  sperm/ml, still more preferably about  $1 \times 10^3$  to about  $1 \times 10^6$  sperm/ml, even more preferably about  $1 \times 10^4$  to about  $1 \times 10^5$  sperm/ml, and most preferably about  $1 \times 10^5$  sperm/ml. In an alternative embodiment, buffer may be 15 added to achieve an "intermediate" concentration of sperm in the sperm suspension, i.e., buffer is added to achieve a sperm suspension that contains about  $1 \times 10^7$  to about  $1 \times 10^8$  sperm/ml. In yet another alternative embodiment, buffer may be added to achieve a "relatively high" 20 concentration of sperm in the sperm suspension, i.e., buffer is added to achieve a sperm suspension that contains greater than about  $1 \times 10^8$  sperm/ml, preferably about  $1 \times 10^8$  to about  $5 \times 10^{10}$  sperm/ml, more preferably about  $1.5 \times 10^8$  to about  $2 \times 10^{10}$  sperm/ml, even more preferably 25 about  $1.5 \times 10^8$  to about  $2 \times 10^8$  sperm/ml, and still more preferably about  $1.5 \times 10^8$  sperm/ml.

[0036] An additional consideration in determining the amount of buffer employed, i.e., whether the buffered sperm suspension will have a "relatively low," an "intermediate," 30 or a "relatively high" concentration of sperm in the sperm suspension, includes the method by which the sperm cells may be subsequently sorted or enriched. For example, the sperm cells may be sorted using flow cytometry as described

in greater detail below. In such an instance, the buffered sperm suspension may typically be of an "intermediate" or "relatively high" concentration of sperm/ml. Other sorting or enrichment techniques may benefit from a lesser 5 concentration of sperm cells, such as a "relatively low" concentration of sperm cells, labeled with a marker, such as for example the dyes and labels described herein.

[0037] Alternatively, the sperm may be combined with an inhibitory buffer to form an inhibited sperm suspension. 10 Inhibitory buffers cause the sperm cells to emulate sperm cells of the epididymis of a mammal, such as for example a bull, by simulating the fluid environment of the epididymis or epididymal tract of the mammal. Such a buffer would reduce or inhibit the motility or metabolic activity of the 15 sperm. Exemplary buffers of this class include carbonate based buffers, such as for example those disclosed in Salisbury & Graves, J. Reprod. Fertil., 6:351-359 (1963). A preferred buffer of this type comprises 0.204g NaHCO<sub>3</sub>, 0.433g KHCO<sub>3</sub>, and 0.473g C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>·H<sub>2</sub>O per 25mL of purified 20 water (0.097 moles/L of NaHCO<sub>3</sub>, 0.173 moles/L of KHCO<sub>3</sub>, 0.090 moles/L C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>·H<sub>2</sub>O in water). In addition, the inhibited sperm suspension may also contain a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly.

25 [0038] In addition to a buffer, the sperm suspension may also contain a range of additives to enhance sperm viability or motility or to provide other benefits. Exemplary additives include energy sources, protein sources, and antibiotics. One or more of these additives 30 may be introduced into the buffer or buffered solution before the formation of the buffered sperm suspension or, alternatively, may be separately introduced into the sperm suspension.

[0039] One or more energy sources may be added to minimize or inhibit the sperm cells from oxidizing intracellular phospholipids and other cellular components. Exemplary energy sources include monosaccharides, such as 5 fructose, glucose, galactose and mannose, and disaccharides, such as sucrose, lactose, maltose, and trehalose, as well as other polysaccharides. For example, the resulting sperm suspension may include about 1% (w/v) to about 4% (w/v) of the energy source(s). If included, 10 the energy source is preferably fructose and the sperm suspension contains about 2.5% (w/v).

[0040] To minimize dilution shock, provide support to the cells, or disperse the cells throughout the suspension, a protein source may also be included in the buffer, 15 buffered solution, sperm suspension, or buffered sperm suspension. Exemplary protein sources include egg yolk, egg yolk extract, milk (including heat homogenized and skim), milk extract, soy protein, soy protein extract, serum albumin, bovine serum albumin, human serum substitute 20 supplement, and combinations thereof. Albumin, and more particularly bovine serum albumin (BSA), is a preferred protein source. For example, if included, BSA may be present in the sperm suspension in an amount of less than about 5.0% (w/v), preferably less than about 2% (w/v), more 25 preferably less than about 1% (w/v), and most preferably in an amount of about 0.1% (w/v).

[0041] The use of a protein source, such BSA, alone may initiate the process of capacitation in a percentage of the sperm cells in the suspension. It is preferred that 30 this process take place in the female reproductive tract. Therefore, in order to inhibit the initiation of capacitation during dilution, as well as during the subsequent staining and sorting, an alternative protein

source or a protein substitute may be included in the sperm suspension. The alternative protein source or protein substitute possess the advantageous effects of a typical protein source, such as BSA, in addition to the ability to  
5 inhibit the initiation of capacitation in a larger percentage of the cells in the sperm suspension. Examples of an alternative protein source include human serum substitute supplement (SSS) (Irvine Scientific, Santa Ana, CA) and cholesterol enhanced BSA, while an example of a  
10 protein substitute includes a polyvinyl alcohol, such as for example, a low to medium viscosity polyvinyl alcohol generally of a molecular weight of about 30,000 to about 60,000. Generally, if included, these compositions will be present in the same amounts as disclosed above with respect  
15 to BSA, with the total albumin content of the buffer or buffered solution generally not exceeding about 5.0% (w/v).

[0042] An antibiotic may be added to the sperm suspension or buffered sperm suspension in order to inhibit bacterial growth. Exemplary antibiotics include, for  
20 example, tylosin, gentamicin, lincomycin, spectinomycin, Linco-Spectin® (lincomycin hydrochloride-spectinomycin), penicillin, streptomycin, ticarcillin, or any combination thereof. The antibiotics may be present in a concentration of about 50 $\mu$ g to about 800 $\mu$ g per ml of semen, regardless of  
25 whether the semen is neat, buffered, or contains additional substances, such as for example, any of the additives mentioned herein. The Certified Semen Services (CSS) and National Association of Animal Breeders (NAAB) have promulgated guidelines regarding the use of antibiotics  
30 with respect to sperm collection and use.

***Staining of the cells***

[0043] A composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly may be used in the process of staining the 5 cells. Generally, a process of staining sperm cells may comprise the formation of a staining mixture, sometimes referred to as a labeling mixture, containing intact viable sperm cells, a composition which regulates oxidation/reduction reactions intracellularly and/or 10 extracellularly, and a dye, sometimes referred to as a label. In this aspect of the invention, the composition may be contacted with the sperm cells to form a sperm suspension, and then the suspension contacted with a DNA 15 selective dye. In this embodiment, the sperm source may be neat semen, or alternatively, a sperm-containing semen derivative obtained by centrifugation or the use of other means to separate semen into fractions.

[0044] Once obtained, the sperm cells may be introduced into the staining mixture in the form of neat 20 semen or in the form of a suspension derived therefrom, e.g., a sperm suspension or a buffered sperm suspension as discussed above with respect to collection of the cell sample.

[0045] The dye may be in the form of a neat solid or a 25 liquid composition. The dye may also be dissolved or dispersed in an unbuffered liquid to form a dye solution. Alternatively, the dye may be in the form of a dye suspension comprising a dye and a buffer or buffered 30 solution that is biologically compatible with sperm cells. A range of exemplary buffers and buffered solutions are discussed above with respect to sample collection. For example, among the buffers which may be used is a TCA buffer solution comprising 3% TRIS base, 2% citric acid

monohydrate, and 1% fructose in water at a pH of about 7.0, or a carbonate-based buffer solution comprising 0.204g NaHCO<sub>3</sub>, 0.433g KHCO<sub>3</sub>, and 0.473g C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>·H<sub>2</sub>O per 25mL of purified water (0.097 moles/L of NaHCO<sub>3</sub>, 0.173 moles/L of 5 KHCO<sub>3</sub>, 0.090 moles/L C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>·H<sub>2</sub>O in water). Thus, for example, a staining mixture may be formed by combining neat semen with a dye and a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly. Alternatively, the staining mixture may 10 be formed by combining neat semen with a buffer or buffered solution, a dye, and a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly. Additionally, the staining mixture may be formed by combining a sperm suspension with a dye.

15 [0046] The staining mixture may be formed by using one or more UV or visible light excitable, DNA selective dyes as previously described in U.S. Patent No. 5,135,759 and WO 02/41906. Exemplary UV light excitable, selective dyes include Hoechst 33342 and Hoechst 33258, each of which is 20 commercially available from Sigma-Aldrich (St. Louis, MO). Exemplary visible light excitable dyes include SYBR-14, commercially available from Molecular Probes, Inc. (Eugene, OR) and bisbenzimid-BODIPY<sup>®</sup> conjugate 6-{[3-((2Z)-2-{[1-(difluoroboryl)-3,5-dimethyl-1H-pyrrol-2-yl]methylene}-2H-pyrrol-5-yl)propanoyl]amino}-N-[3-(methyl{3-[(4-[6-(4- 25 methylpiperazin-1-yl)-1H,3'H-2,5'-bibenzimidazol-2'-yl]phenoxy}acetyl)amino]propyl]amino]propyl]hexanamide ("BBC") described in WO 02/41906. Each of these dyes may be used alone or in combination; alternatively, other cell 30 permeant UV and visible light excitable dyes may be used, alone or in combination with the aforementioned dyes, provided the dye does not detrimentally affect the viability of the sperm cells to an unacceptable degree when

used in concentrations which enable sorting as described elsewhere.

[0047] Alternatively, the staining mixture may be formed using fluorescent polyamides, and more specifically 5 polyamides with a fluorescent label or reporter conjugated thereto. Such labels will fluoresce when bound to nucleic acids. Examples of polyamides with a fluorescent label or reporter attached thereto include, for example, those disclosed in Best et al., *Proc. Natl. Acad. Sci. USA*, 10 100(21): 12063-12068 (2003); Gygi, et al., *Nucleic Acids Res.*, 30(13): 2790-2799 (2002); U.S. Patent No. 5,998,140; U.S. Patent No. 6,143,901; and U.S. Patent No. 6,090,947, the content of each of which is hereby incorporated herein by reference.

15 [0048] Fluorescent nucleotide sequences may also be used to label the sperm cells. Such nucleotide sequences fluoresce when hybridized to a nucleic acid containing a target or complementary sequence, but are otherwise non-fluorescent when in a non-hybridized state. Such 20 oligonucleotides are disclosed, for example, in U.S. Patent Application Publication No. 2003/0113765 (hereby incorporated herein by reference).

[0049] Sex specific antibodies may also be used to label the sperm cells in a staining mixture. In this 25 embodiment, for example, a sex specific antibody may be conjugated with a fluorescent moiety (or equivalent reporter molecule). Because the antibody binds to antigens present on only an X chromosome-bearing or, alternatively, a Y chromosome-bearing cell, such cells can be selectively 30 identified based upon their fluorescence (versus the non-fluorescence of an unlabeled cell). Moreover, more than one sex specific antibody, each antibody having a different fluorescent moiety attached thereto, may be used

simultaneously. This allows for differentiation of X chromosome-bearing and Y chromosome-bearing cells based upon the differing fluorescence of each.

[0050] Luminescent, color-selective nanocrystals may 5 also be used to label sperm cells in a staining mixture. Also referred to as quantum dots, these particles are well known in the art, as demonstrated by U.S. Patent No. 6,322,901 and U.S. Patent No. 6,576,291, each of which is hereby incorporated herein by reference. These 10 nanocrystals have been conjugated to a number of biological materials, including for example, peptides, antibodies, nucleic acids, streptavidin, and polysaccharides, (see, for example, U.S. Patent Nos. 6,207,392; 6,423,551; 5,990,479, and 6,326,144, each of which is hereby incorporated herein 15 by reference), and have been used to detect biological targets (see, for example, U.S. Patent Nos. 6,207,392 and 6,247,323, each of which is hereby incorporated herein by reference).

[0051] The preferred concentration of the DNA 20 selective dye in the staining mixture is a function of a range of variables which include the permeability of the cells to the selected dye, the temperature of the staining mixture, the amount of time allowed for staining to occur, and the degree of enrichment desired in the subsequent 25 sorting step. In general, the dye concentration is preferably sufficient to achieve the desired degree of staining in a reasonably short period of time without substantially detrimentally affecting sperm viability. For example, the concentration of Hoechst 33342, Hoechst 33258, 30 SYBR-14, or BBC in the staining mixture will generally be between about 0.1 $\mu$ M and about 1.0M, preferably from about 0.1 $\mu$ M to about 700 $\mu$ M, and more preferably from about 100 $\mu$ M to about 200 $\mu$ M. In a particularly preferred embodiment,

the concentration of Hoechst 33342, Hoechst 33258, SYBR-14, or BBC in the staining mixture will generally be between about 400 $\mu$ M to about 500 $\mu$ M, and most preferably about 450 $\mu$ M. Accordingly, under one set of staining conditions, 5 the concentration of Hoechst 33342 is preferably about 100 $\mu$ M. Under another set of staining conditions, the concentration of Hoechst 33342 is about 150 $\mu$ M. Under still another set of staining conditions the concentration is preferably about 200 $\mu$ M. Under yet another set of staining 10 conditions the concentration of Hoechst 33342 is most preferably about 450 $\mu$ M.

[0052] As another example, the concentration of a fluorescent polyamide, such as for example, those described in U.S. Application Publication No. 2001/0002314, will 15 generally be between about 0.1 $\mu$ M and about 1mM, preferably from about 1 $\mu$ M to about 1mM, more preferably about 5 $\mu$ M to about 100 $\mu$ M, even more preferably about 10 $\mu$ M.

[0053] In addition to buffer, other additives may be included in the staining mixture to enhance the viability 20 or motility of the sperm; these additives may be provided as part of the sperm source, the dye source, or separately to the staining mixture. Such additives include energy sources, antibiotics, and seminal plasma, the first two of which are discussed above with respect to collection of the 25 cell sample, and the last of which is discussed below with respect to collection of the sorted cells. Such additives may be added during the staining techniques in accordance therewith.

[0054] The staining mixture may be maintained at any 30 of a range of temperatures; typically, this will be within a range of about 4°C to about 50°C. For example, the staining mixture may be maintained at a "relatively low" temperature, i.e., a temperature of about 4°C to about

30°C; in this embodiment, the temperature is preferably from about 20°C to about 30°C, more preferably from about 25°C to about 30°C, and most preferable at about 28°C. Alternatively, the staining mixture may be maintained 5 within an "intermediate" temperature range, i.e., a temperature of about 30°C to about 39°C; in this embodiment, the temperature is preferably at about 34°C to about 39°C, and more preferably about 37°C. In addition, the staining mixture may be maintained within a "relatively 10 high" temperature range, i.e., a temperature of about 40°C to about 50°C; in this embodiment, the temperature is preferably from about 40°C to about 45°C, more preferably from about 40°C to about 43°C, and most preferably at about 41°C. Selection of a preferred temperature generally 15 depends upon a range of variables, including for example, the permeability of the cells to the dye(s) being used, the concentration of the dye(s) in the staining mixture, the amount of time the cells will be maintained in the staining mixture, and the degree of enrichment desired in the 20 sorting step.

[0055] Uptake of dye by the sperm cells in the staining mixture is allowed to continue for a period of time sufficient to obtain the desired degree of DNA staining. That period is typically a period sufficient for 25 the dye to bind to the DNA of the sperm cells such that X and Y chromosome-bearing sperm cells may be sorted based upon the differing and measurable fluorescence intensity between the two. Generally, this will be no more than about 160 minutes, preferably no more than about 90 minutes, still more preferably no more than about 60 30 minutes, and most preferably from about 5 minutes to about 40 minutes.

[0056] Accordingly, in one embodiment, a staining mixture is formed comprising sperm cells, a dye in a concentration from about 100 $\mu$ M to about 200 $\mu$ M, and a composition which regulates oxidation/reduction reactions 5 intracellularly and/or extracellularly, and the staining mixture is held for a period of time at a temperature of about 41°C. In another embodiment, the composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly comprises pyruvate in a 10 concentration of about 10mM, vitamin K in a concentration of about 100 $\mu$ M, or lipoic acid in a concentration of about 1mM.

[0057] In still another embodiment, a staining mixture is formed comprising sperm cells, a dye in a concentration 15 from about 100 $\mu$ M to about 200 $\mu$ M, and a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly, and the staining mixture is held for a period of time at a temperature of about 28°C. In another embodiment, the a composition which regulates 20 oxidation/reduction reactions intracellularly and/or extracellularly comprises pyruvate in a concentration of about 10mM, vitamin K in a concentration of about 100 $\mu$ M, or lipoic acid in a concentration of about 1mM.

[0058] In yet another example, a staining mixture is 25 formed comprising sperm cells, a buffer comprising 0.204g NaHCO<sub>3</sub>, 0.433g KHCO<sub>3</sub>, and 0.473g C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>·H<sub>2</sub>O per 25mL of purified water (0.097 moles/L of NaHCO<sub>3</sub>, 0.173 moles/L of KHCO<sub>3</sub>, 0.090 moles/L C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>·H<sub>2</sub>O in water), a dye in a concentration from about 100 $\mu$ M to about 200 $\mu$ M, and a 30 composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly, and the staining mixture is held for a period of time at a temperature of about 28°C. In another embodiment, the staining mixture is

held for a period of time at a temperature of about 41°C. In yet another embodiment, the composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly comprises pyruvate in a concentration of 5 about 10mM, vitamin K in a concentration of about 100μM, or lipoic acid in a concentration of about 1mM.

**Sorting**

[0059] A composition which regulates oxidation/reduction reactions intracellularly and/or 10 extracellularly may also be used during sorting of the sperm cells. Generally, once the sperm are stained according to the present invention, they may be sorted according to any known means that allows for separation based upon fluorescence. Commonly used and well known 15 methods include flow cytometry systems, as exemplified by and described in U.S. Patent Nos. 5,135,759, 5,985,216, 6,071,689, 6,149,867, and 6,263,745, International Patent Publications WO 99/33956 and WO 01/37655, U.S. Patent Application Serial No. 10/812,351 and corresponding 20 International Patent Publication WO 2004/088283.

[0060] According to the above-referenced flow cytometry methods, the stained cells are introduced as a sample fluid into the nozzle of a flow cytometer as described in Exhibit A. In one embodiment, therefore, the 25 sample fluid may comprise the stained sperm cells and a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly.

[0061] The sample fluid is typically surrounded by a sheath fluid. The sheath fluid permits the sperm cells in 30 the sample fluid to be drawn out into a single file line. The sheath fluid is collected along with the sperm cells by the collection system of the flow cytometer and therefore

forms part of the post-sort environment for the sperm cells. Thus, it is desirable that the sheath fluid provides a protective effect to the cells upon contact of cells by the sheath fluid.

5 [0062] Accordingly, the sheath fluid generally comprises a buffer or buffered solution and a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly. Examples of buffers and buffered solutions and illustrative 10 concentrations of the same that may be used in the sheath fluid are disclosed above with respect to sample collection and dilution. In a particular embodiment, the sheath fluid comprises 0.96% Dulbecco's phosphate buffered saline (w/v), 0.1% BSA (w/v), in water at a pH of about 7.0.

15 [0063] Optionally, the sheath fluid may also contain a range of additives that are beneficial to sperm viability or motility. Such additives include, for example, an energy source, a protein source, an antibiotic, and polyvinyl alcohol. Each of these additives, and examples 20 of the same, is discussed above with respect to collection of the cell sample. Such additives may be added to the sheath fluid in accordance therewith.

[0064] The sheath fluid may optionally be filtered prior to the sorting step. Contaminants that may be 25 present in the sheath fluid, such as non-soluble particulates, may interfere with sorting. Therefore, the sheath fluid may be filtered prior to its introduction into a flow cytometer. Such filters and methods of using the same are well known in the art. Generally, the filter is a 30 membrane of about 0.1 microns to about 0.5 microns, preferably about 0.2 microns to about 0.3 microns, and more preferably about 0.2 microns.

[0065] The stained cells may be introduced into the sheath fluid at any time subsequent to staining. Typically, a stream of the stained cells in the sample fluid is injected into a stream of sheath fluid within the 5 nozzle of the flow cytometer. Initially, there is substantially no contacting of the sample fluid and the sheath fluid due to laminar flow of the fluids as discussed in more detail below. It is desirable that the sample fluid and the sheath fluid remain as substantially discrete 10 flowing streams until after the particles (e.g., the stained sperm cells) in the sample fluid have been analyzed. At some point, however, the sheath fluid and the cells of the sample fluid come in contact with one another. For instance in a droplet sorting flow cytometer (discussed 15 below) the sheath fluid and sample fluid begin contacting one another as droplets are being formed downstream of the interrogation location.

[0066] At the time of the introduction of the stained cells and the sheath fluid, both the stained cells and the 20 sheath fluid may be at a temperature from about 4°C to about 50°C. The sheath fluid and the stained cells may be at the same or at different temperatures, with either being at a higher temperature than the other. Accordingly, in one embodiment, at the time of the introduction of the 25 stained cells and the sheath fluid, both the cells and the sheath fluid are at the same temperature; for example, at a "relatively low" temperature, such as for example at about 5°C to about 8°C; at an "intermediate" temperature, such as for example at about 25°C to about 30°C; or at a 30 "relatively high" temperature, such as for example at about 40°C to about 43°C. In another embodiment, the stained cells are at a higher temperature than the sheath fluid, such as for example, the cells being at about 40°C to about

43°C and the sheath fluid being at about room temperature or at about 5°C. In yet another embodiment, the stained cells are at a lower temperature than the sheath fluid.

***Collection of the sorted cells***

5 [0067] Once sorted, the sorted cells are collected in a vessel that contains a collection fluid. Generally, the purpose of the collection fluid includes cushioning the impact of the sperm cells with the collection vessel or providing a fluid support for the cells. Accordingly, the 10 collection fluid may comprise a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly, a buffer or buffered solution, and a protein source.

15 [0068] If included, examples of buffers or buffered solutions that may be used in the collection fluid are disclosed above with respect to collection of the sample cells. Typically, these buffers or buffer solutions will be in a concentration of about 0.001M to about 1.0M and have a pH of about 4.5 to about 8.5, preferably of about 20 5.0 to about 8.0, more preferably of about 5.5 to about 7.5, still more preferably of about 6.0 to about 7.0, even more preferably of about 6.5 to about 7.0, and most preferably of about 6.5. In one embodiment, the collection fluid contains buffer comprising 0.96% Dulbecco's PBS (w/v) 25 at a pH of about 7.0. In another embodiment, the collection fluid contains buffer comprising 0.96% Dulbecco's PBS (w/v) at a pH of about 6.5. In another embodiment, the collection fluid contains a buffer solution comprising 0.204g NaHCO<sub>3</sub>, 0.433g KHCO<sub>3</sub>, and 0.473g C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>·H<sub>2</sub>O 30 per 25mL of purified water (0.097 moles/L of NaHCO<sub>3</sub>, 0.173 moles/L of KHCO<sub>3</sub>, 0.090 moles/L C<sub>6</sub>H<sub>8</sub>O<sub>7</sub>·H<sub>2</sub>O in water).

[0069] If included, the protein source may be any protein source that does not interfere with the viability of the sperm cells and is compatible with the particular buffer or buffered solution being used. Examples of common 5 protein sources include milk (including heat homogenized and skim), milk extract, egg yolk, egg yolk extract, soy protein and soy protein extract. Such proteins may be used in a concentration from about 1% (v/v) to about 30% (v/v), preferably from about 10% (v/v) to about 20% (v/v), and 10 more preferably about 10% (v/v). While milk may be used in combination with a buffer or buffered solution, generally milk is used in the absence of the same, as milk is a solution itself that may serve the same purpose of a buffer or buffered solution. In such instances, the collection 15 fluid may contain about 80% (v/v) to about 90% (v/v) milk.

[0070] In addition to or in lieu of the protein source, the collection fluid may also comprise seminal plasma. Seminal plasma serves the dual benefits of improving sperm viability and motility and of stabilizing 20 the sperm membrane (thereby preventing capacitation during the collection and storage of the sperm). Maxwell et al., *Reprod. Fert. Dev.* (1998) 10: 433-440. The seminal plasma may be from the same mammal from which the semen sample was obtained, from a different mammal of the same species, or 25 from a mammal of a different species. If included in the collection fluid, typically the percentage of seminal plasma will be in the range of about 0.5% (v/v) to about 10% (v/v). If used in combination with a protein source, such as for example egg yolk or milk, the total percentage 30 of seminal plasma and protein source will range from about 1% (v/v) to about 30% (v/v). In such instances, the percentage of seminal plasma will be inversely proportional to the percentage of the protein source. Accordingly, in

one embodiment, the collection fluid comprises seminal plasma. In another embodiment, the collection fluid contains seminal plasma in an amount of about 0.5% (v/v) to about 10% (v/v), preferably in an amount of about 4% (v/v) 5 to about 6% (v/v), and more preferably in an amount of about 5% (v/v). In another embodiment, the collection fluid contains a protein source and seminal plasma. In yet another embodiment, the collection fluid comprises seminal plasma and egg yolk, the percentage of both totaling 10 between about 1% (v/v) and about 30% (v/v).

[0071] Optionally, the collection fluid may also contain a range of additives that are beneficial to sperm viability or motility. Examples of such additives include an energy source and an antibiotic, each of which is 15 discussed above with respect to collection of the sample cells. Such additives may be added to the collection fluid in accordance therewith.

[0072] Accordingly, in a certain embodiment, the collection fluid comprises A composition which regulates 20 oxidation/reduction reactions intracellularly and/or extracellularly, 0.96% Dulbecco's PBS (w/v), 1% (w/v) fructose, 10% (v/v) egg yolk in water, at a pH of about 7.0. In yet another embodiment, the composition which regulates oxidation/reduction reactions intracellularly 25 and/or extracellularly comprises 10mM pyruvate, 100 $\mu$ M vitamin K, 1mM of lipoic acid, or any combination thereof.

[0073] Alternatively, and in lieu of the use of a collection fluid, the sorted cells may be collected into a vessel containing or coated with a cryoextender used in the 30 subsequent cryopreservation steps and further described below. Accordingly, in one particular embodiment, the sorted cells are collected into a cryoextender. For example, the cryoextender may comprise water, Triladyl®

(Minitube, Verona, WI, comprising glycerol, tris, citric acid, fructose, 5mg/100ml tylosin, 25mg/100ml gentamycin, 30mg/100ml Spectinomycin, and 15mg/100ml Lincomycin), egg yolk, and a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly. In yet another embodiment, the cryoextender comprises 25g Triladyl®, and 25g egg yolk per 75ml of water, and composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly.

10 [0074] It is to be understood that the percent concentrations of protein in the collection fluid disclosed herein are those prior to the addition of the flow sorted cells. The addition of the flow sorted cells may dilute the final concentration of the collection fluid to about 1/20 that of what it was prior to the addition of the flow sorted cells. Therefore, for example, the collection fluid may initially contain about 10% (v/v) egg yolk. After the flow sorted cells are collected in the collection vessel containing the collection fluid, the final concentration of egg yolk will be reduced to about 0.5% (v/v). Alternatively, the addition of the flow sorted cells may dilute the final concentration of the collection fluid to about 1/15 that of what it was prior to the addition of the flow sorted cells. Therefore, for example, the collection fluid may initially contain about 20% (v/v) egg yolk. After the flow sorted cells are collected in the collection vessel containing the collection fluid, the final concentration of egg yolk will be reduced to about 1.3% (v/v).

***Cryoextension of the sorted cells***

[0075] Once the sperm have been sorted and collected in the collection vessels, they may be used for inseminating female mammals. This can occur almost 5 immediately, requiring little additional treatment of the sperm. Likewise, the sperm may also be cooled or frozen for use at a later date. In such instances, the sperm may benefit from additional treatment to minimize the impact upon viability or post-thaw motility as a result of cooling 10 and freezing.

[0076] Generally, a cryoextender comprises a buffer or buffered solution, a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly, a protein source, and a cryoprotectant. 15 Examples of buffers and buffered solutions that may be used in the cryoextender are disclosed above with respect to sample collection and extension. Typically, these buffers will be in a concentration of about 0.001M to about 1.0M and have a pH of about 4.5 to about 8.5, preferably of 20 about 7.0.

[0077] If included, a protein source may be added, for example, to provide support to the cells. The protein source may be any protein source that does not interfere with the viability of the sperm cells and is compatible 25 with the particular buffer or buffered solution being used. Examples of common protein sources include milk (including heat homogenized and skim), milk extract, egg yolk, egg yolk extract, soy protein and soy protein extract. Such proteins may be found in a concentration from about 30 10% (v/v) to about 30% (v/v), preferably from about 10% (v/v) to about 20% (v/v), and more preferably about 20% (v/v). While milk may be used in combination with a buffer or buffered solution, generally milk is used in the

absence of the same, as milk is a solution itself that may serve the same purpose of a buffer or buffered solution. In such instances, the cryoextender would contain about 80% (v/v) to about 90% (v/v) milk.

5 [0078] A cryoprotectant is preferably included in the cryoextender to lessen or prevent cold shock or to maintain fertility of the sperm. Numerous cryoprotectants are known in the art. Selection of a cryoprotectant suitable for use with a given extender may vary, and depends upon the

10 species from which the sperm to be frozen were obtained. Examples of suitable cryoprotectants include, for example, glycerol, dimethyl sulfoxide, ethylene glycol, propylene glycol, trehalose, Triladyl<sup>®</sup> and combinations thereof. If included, generally, these cryoprotectants are present in

15 the cryoextender in an amount of about 1% (v/v) to about 15% (v/v), preferably in an amount of about 5% (v/v) to about 10% (v/v), more preferably in an amount of about 7% (v/v), and most preferably in an amount of about 6% (v/v).

20 [0079] In one particular embodiment, the cryoextender comprises water, Triladyl<sup>®</sup>, egg yolk, and a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly. In yet another embodiment, the cryoextender comprises 25g Triladyl<sup>®</sup> and 25g

25 egg yolk per 75 ml of water, and a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly.

[0080] Optionally, the cryoextender may also contain a range of additives that are beneficial to sperm viability or motility and that prevent or lessen the detrimental side effects of cryopreservation. Such additives may include, for example, an energy source or an antibiotic, each of which is discussed above with respect to sample collection

and dilution. Such additives may be added to the cryoextender in accordance therewith.

[0081] Having described the invention in detail, it will be apparent that modifications and variations are 5 possible without departing the scope of the invention defined in the appended claims.

**EXAMPLES**

[0082] The following non-limiting examples are provided to further illustrate the present invention.

10

**Example 1**

[0083] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample diluted in 2 parts carbonate buffer for transportation at 25°C in a 15 temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. *Theriogenology*, 49(4): 20 871-9 (Mar 1998)). Based on the semen concentration, 1mL of 150 X 10<sup>6</sup> sperm/ml suspension was prepared by removing an aliquot of the carbonate sperm suspension centrifuging the sperm suspension at 500 X g for 5 minutes, removing the supernatant and re-suspending the pellet in 41°C TCA buffer 25 pH 7.3. An additional 1mL of 150 X 10<sup>6</sup> sperm/ml was prepared by suspending an aliquot of semen in 41°C TCA buffer containing 10mM pyruvate at pH 7.3. To the sperm suspensions, aliquots of a 10mM Hoechst solution in water were added to yield the dye concentration of 400μM Hoechst. 30 The sperm suspensions were maintained in a 41°C water bath for the duration of the staining period. Sperm suspensions were analyzed by removing a 50μL aliquot from the staining

sperm suspension, adding 200 $\mu$ L of the same buffer at the same temperature and analyzing by IVOS to measure % progressive motility (% Prog Mot). Results of the IVOS analysis are summarized in Figure 1.

5 **Example 2**

[0084] Sperm samples were obtained and prepared in the same manner as in Example 1 with the following exception. The buffer used to suspend the sperm for staining and IVOS analysis were TCA and TCA containing 10uM Vitamin K.

10 Results of the IVOS analysis are summarized in Figure 2.

**Example 3**

[0085] Sperm samples were obtained and prepared in the same manner as in Example 1 with the following exception.

15 The buffer used to suspend the sperm for staining and IVOS analysis were TCA and TCA containing 100uM Vitamin K.

Results of the IVOS analysis are summarized in Figure 3.

**Example 4**

[0086] Sperm samples were obtained and prepared in the same manner as in Example 1 with the following exception.

The buffers used to suspend the sperm for staining and IVOS analysis were TCA and TCA containing 1mM Lipoic Acid.

Results of the IVOS analysis are summarized in Figure 4.

**Example 5**

25 [0087] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample diluted in 2 parts carbonate buffer for transportation at 25°C in a temperature-controlled container to the staining facility.

Upon receipt, the semen was analyzed for concentration,

30 motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. *Theriogenology*, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, 1mL

of  $150 \times 10^6$  sperm/ml suspension was prepared by centrifuging the sperm suspension at  $500 \times g$  for 5 minutes, removing the supernatant and re-suspending the pellet in  $28^{\circ}\text{C}$  TCA buffer pH 7.3. An additional 1mL of  $150 \times 10^6$  sperm/ml was prepared by suspending an aliquot of semen in  $28^{\circ}\text{C}$  TCA buffer containing 10mM pyruvate at pH 7.3. To the sperm suspensions, aliquots of a 10 mM Hoechst solution in water were added to yield the dye concentration of  $600\mu\text{M}$  Hoechst. The sperm suspensions were maintained in  $28^{\circ}\text{C}$  water bath for the duration of the staining period. Sperm suspensions were analyzed by removing a  $50\mu\text{L}$  aliquot from the staining sperm suspension, adding  $200\mu\text{L}$  of the same buffer at the same temperature and analyzing by IVOS to measure percent progressive motility (% Prog Mot). Results of the IVOS analysis are summarized in Figure 5.

**Example 6**

[0088] Sperm samples were obtained and prepared in the same manner as in Example 5 with the following exception. The buffer used to suspend the sperm for staining and IVOS analysis were TCA and TCA containing  $100\mu\text{M}$  Vitamin K. Results of the IVOS analysis are summarized in Figure 6.

**Example 7**

[0089] Sperm samples were obtained and prepared in the same manner as in Example 5 with the following exception. The buffer used to suspend the sperm for staining and IVOS analysis were TCA and TCA containing 1mM Lipoic Acid. Results of the IVOS analysis are summarized in Figure 7.

**Example 8**

[0090] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample diluted in 2 parts carbonate buffer for transportation at  $25^{\circ}\text{C}$  in a temperature-controlled container to the staining facility.

Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. *Theriogenology*, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, 1mL of  $150 \times 10^6$  sperm/ml suspensions were prepared by removing an aliquots of the carbonate sperm suspension, centrifuging the sperm suspension at  $500 \times g$  for 5 minutes, removing the supernatant and re-suspending the pellet in 1 ml TCA buffer or in 1 ml TCA buffer with 2.5 mM, 10 mM, 25 mM, or 50 mM pyruvate. To the samples was added MON33342 solution to yield the final dye concentrations of  $600 \mu M$ . The suspensions were incubated in a  $28^\circ C$  water bath. Stained sperm suspensions were analyzed by removing a  $50 \mu L$  aliquot from the staining sperm suspension, adding  $200 \mu L$  of the same buffer at the same temperature and analyzing by IVOS to measure percent progressive motility (% Prog Mot). IVOS results for % Prog Mot are shown in Figures 8.

**Example 9**

[0091] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample diluted in 2 parts carbonate buffer for transportation at  $25^\circ C$  in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. *Theriogenology*, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, 1mL of  $150 \times 10^6$  sperm/ml suspension in TCA buffer was prepared by removing an aliquot of the carbonate sperm suspension, centrifuging the sperm suspension at  $500 \times g$  for 5 minutes, removing the supernatant and re-suspending the pellet in

1mL TCA buffer. 1ml of  $150 \times 10^6$  sperm/ml suspension in 10mM pyruvate in TCA was prepared by removing an aliquot of the carbonate sperm suspension, centrifuging the sperm suspension at 500 X g for 5 minutes, removing the supernatant and re-suspending the pellet in 1mL of 10 mM pyruvate TCA buffer. To samples was added SYBR 14 dye solution to yield the final dye concentrations of 20 $\mu$ M. The suspensions were incubated in a 28°C water bath. Sperm suspensions were analyzed by removing a 50 $\mu$ L aliquot from the staining sperm suspension, adding 200 $\mu$ L of the same buffer at the same temperature and analyzing by IVOS to measure percent progressive motility (% Prog Mot). IVOS results for % Prog Mot are shown in Figures 9.

15 **Example 10**

[0092] Bull semen was collected from a sexually mature bull using an artificial vagina and the sample diluted in 2 parts carbonate buffer for transportation at 25°C in a temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. *Theriogenology*, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, 1mL of  $150 \times 10^6$  sperm/ml suspension in TCA buffer was prepared by removing an aliquot of the carbonate sperm suspension, centrifuging the sperm suspension at 500 X g for 5 minutes, removing the supernatant and re-suspending the pellet in 1mL TCA buffer. 1ml of  $150 \times 10^6$  sperm/ml suspension in 10mM pyruvate in TCA was prepared by removing an aliquot of the carbonate sperm suspension, centrifuging the sperm suspension at 500 X g for 5 minutes, removing the

supernatant and re-suspending the pellet in 1ml of 10 mM pyruvate TCA buffer. To the samples was added BBC solution to yield the final dye concentrations of 100 $\mu$ M. The suspensions were incubated in a 28°C water bath. Stained 5 sperm suspensions were analyzed by removing a 50 $\mu$ L aliquot from the staining sperm suspension, adding 200 $\mu$ L of the same buffer at the same temperature and analyzing by IVOS to measure percent progressive motility (% Prog Mot). IVOS results for % Prog Mot are shown in Figure 10.

10

**Example 11**

[0093] Sperm samples were obtained and prepared in the same manner as in Example 10 with the following exception. The staining concentration was 200 $\mu$ M BBC. Results of the 15 IVOS analysis are summarized in Figure 11.

**Example 12**

[0094] Bull semen was collected from a sexually mature bull using an artificial vagina and transported at 25°C in a 20 temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. *Theriogenology*, 49(4): 25 871-9 (Mar 1998)). Based on the semen concentration, several tubes of 150 X 10<sup>6</sup> sperm/ml suspensions were prepared by suspending semen in a TCA buffer or a carbonate-based inhibitor. Table I. below illustrates the compositions and staining conditions used.

Table I.

Sample Name	Composition	pH	Conc. (uM) Hoechst	Temperature (°C)
10mM pyr TCA	10mM pyruvate in TCA	7.3	600μM	28°C
10mM pyr CO <sub>2</sub>	10mM pyruvate in TCA blanket with CO <sub>2</sub> balloon	7.3	600μM	28°C
Carbonate 6.2	Carbonate based inhibitor, pH 6.2	6.2	600μM	28°C
Carbonate 7.3	Carbonate based inhibitor, pH 7.3	7.3	600μM	28°C

[0095] To the sperm suspensions, aliquots of a 10mM Hoechst solution in water were added to yield a 5 concentration of 600μM Hoechst. The sperm suspensions were maintained in a 28°C water bath for the duration of the staining period (approximately 1 hour). Sperm suspensions were analyzed by removing a 50μL aliquot from the stained sperm suspension, adding 200μL of 25°C 10mM pyruvate in TCA 10 at pH 7.3 to initiate the reversal of the quiescence, allowing at least a five minute equilibration period, and analyzing by IVOS to measure percent progressive motility (% Prog. Mot.). Comparisons of the IVOS percent progressive motilities are seen in Figures 12-14.

15

#### Example 13

[0096] Bull semen was collected from a sexually mature bull using an artificial vagina and transported at 25°C in a temperature-controlled container to the staining facility. 20 Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well

known procedures (Farrell et al. Theriogenology, 49(4): 871-9 (Mar 1998)). Based on the semen concentration, several tubes of  $450 \times 10^6$  sperm/ml suspensions were prepared by suspending semen in either a TCA buffer or a 5 carbonate based inhibitor. Table II. below illustrates the compositions and staining conditions used.

Table II.

Sample Name	Composition	pH	Conc. (uM) Hoechst	Temperature (°C)
10mM pyr TCA	10mM pyruvate in TCA	7.3	1000uM	28°C
Carbonate 6.2	Carbonate based inhibitor, pH 6.2	6.2	1000uM	28°C
Carbonate 7.3	Carbonate based inhibitor, pH 7.3	7.3	1000uM	28°C

10 [0097] To the sperm suspensions, aliquots of a 10mM Hoechst solution in water were added to yield a concentration of  $1000\mu\text{M}$  Hoechst. The sperm suspensions were maintained in a 28°C water bath for 1 hour, and were then diluted to  $150 \times 10^6$  sperm/ml with 10mM pyruvate in TCA 15 or a carbonate-based inhibitor at a pH 6.2 as specifically indicated in each figure to dilute to a concentration typical for sorting. Sperm suspensions were analyzed by removing a 50uL aliquot from the stained and diluted sperm suspension at the time period designated within each figure 20 and adding 200uL of 25°C 10mM pyruvate in TCA at pH 7.3 to initiate the reversal of the quiescence, allowing at least a five minute equilibration period, and analyzing the aliquot by IVOS to measure the percent progressive motility. Comparisons of the IVOS percent progressive 25 motilities are seen in Figures 15-17.

**Example 14**

[0098] Bull semen was collected from a sexually mature bull using an artificial vagina and transported at 25°C in a 5 temperature-controlled container to the staining facility. Upon receipt, the semen was analyzed for concentration, motility and progressive motility by the Hamilton-Thorn Motility Analyzer (IVOS), according to standard and well known procedures (Farrell et al. Theriogenology, 49(4): 10 871-9 (Mar 1998)). Based on the semen concentration, several tubes of  $450 \times 10^6$  sperm/ml suspensions were prepared by suspending semen in either a TCA buffer or a carbonate based inhibitor. Table II. below illustrates the compositions and staining conditions used.

15 **Table III**

Sample Name	Buffer	pH	Conc (uM) Hoechst	Temperature (°C)
10mM pyr TCA	10mM pyruvate in TCA	7.3	300μM	41°C
Carbonate 6.2	Carbonate based inhibitor, pH 6.2	6.2	300μM	41°C
Carbonate 7.3	Carbonate based inhibitor, pH 7.3	7.3	300μM	41°C

[0099] To the sperm suspensions, aliquots of a 10mM Hoechst solution in water were added to yield a concentration of 300μM Hoechst. The sperm suspensions were 20 maintained in a 41°C water bath for 30 minutes, and then diluted to  $150 \times 10^6$  sperm/ml with 10 mM pyruvate in TCA or a carbonate-based inhibitor at pH 6.2 as specifically indicated in each figure to dilute to a concentration typical for sorting. Sperm suspensions were analyzed by 25 removing a 50μL aliquot from the stained and diluted sperm

suspension at the time period designated within each figure and adding 200 $\mu$ L of 25°C 10mM pyruvate in TCA at pH 7.3 to initiate the reversal of the quiescence, allowing at least a five minute equilibration period, and analyzing by IVOS 5 to measure the percent progressive motility. Comparisons of the IVOS percent progressive motilities are seen in Figures 18-20.

WHAT IS CLAIMED IS:

1. A staining mixture comprising viable spermatozoa, a composition which regulates oxidation/reduction reactions intracellularly and/or extracellularly, and a DNA selective dye, the concentration of the composition in the staining mixture being greater than 50 $\mu$ M when the composition is pyruvate.  
5
2. The staining mixture of claim 1, wherein the composition is selected from the group consisting of pyruvate, vitamin K, lipoic acid, glutathione, flavins, quinones, superoxide dismutase, superoxide dismutase mimics, and any combinations thereof.  
5
3. The staining mixture of claim 2, wherein the composition is selected from the group consisting of pyruvate, vitamin K, lipoic acid, and combinations thereof.
4. The staining mixture of claim 1, wherein the composition comprises pyruvate at a concentration selected from the group consisting of about 2.5mM, about 10mM, about 15mM, about 25mM, and about 50mM.
5. The staining mixture of claim 1, wherein the composition comprises vitamin K at a concentration selected from the group consisting of about 10 $\mu$ M, about 50 $\mu$ M, about 75 $\mu$ M, and about 100 $\mu$ M.
6. The staining mixture of claim 1, wherein the composition comprises lipoic acid at a concentration

selected from the group consisting of about 0.1mM, about 0.5mM, about 0.75mM, about 1.0mM, and about 1.5mM.

7. The staining mixture of claim 1, wherein the DNA selective dye is a DNA selective fluorescent dye.

8. The staining mixture of claim 2, wherein the DNA selective dye is a DNA selective fluorescent dye.

9. The staining mixture of claim 1, wherein the dye is a UV excitable or a visible light excitable dye.

10. The staining mixture of claim 2, wherein the dye is a UV excitable or a visible light excitable dye.

11. The staining mixture of claim 1, wherein the dye is selected from the group consisting of Hoechst 33342, Hoechst 33258, SYBR-14, and bisbenzimide-BODIPY<sup>®</sup> conjugate 6-{[3-((2Z)-2-{[1-(difluoroboryl)-3,5-dimethyl-1H-pyrrol-2-yl]methylene}-2H-pyrrol-5-yl)propanoyl]amino}-N-[3-(methyl{3-[({4-[6-(4-methylpiperazin-1-yl)-1H,3'H-2,5'-bibenzimidazol-2'-yl]phenoxy}acetyl)amino]propyl}amino)propyl]hexanamide.

12. The staining mixture of claim 2, wherein the dye is selected from the group consisting of Hoechst 33342, Hoechst 33258, SYBR-14, and bisbenzimide-BODIPY<sup>®</sup> conjugate 6-{[3-((2Z)-2-{[1-(difluoroboryl)-3,5-dimethyl-1H-pyrrol-2-yl]methylene}-2H-pyrrol-5-yl)propanoyl]amino}-N-[3-(methyl{3-[({4-[6-(4-methylpiperazin-1-yl)-1H,3'H-2,5'-bibenzimidazol-2'-yl]phenoxy}acetyl)amino]propyl}amino)propyl]hexanamide.

13. A process for staining sperm cells, the process comprising forming a staining mixture containing intact viable sperm cells, a composition which regulates 5 oxidation/reduction reactions intracellularly and/or extracellularly, and a DNA selective dye, the concentration of the composition in the staining mixture being greater than 50 $\mu$ M when the composition is pyruvate.

14. The process of claim 13, wherein the DNA-selective dye is selected from the group consisting of Hoechst 33342, Hoechst 33258, SYBR-14, and bisbenzimide-BODIPY<sup>®</sup> conjugate 6-{[3-((2Z)-2-{[1-(difluoroboryl)-3,5-dimethyl-1H-pyrrol-2-5 yl]methylene}-2H-pyrrol-5-yl)propanoyl]amino}-N-[3-(methyl{3-[({4-[6-(4-methylpiperazin-1-yl)-1H,3'H-2,5'-bibenzimidazol-2'-yl]phenoxy}acetyl)amino]propyl}amino)propyl]hexanamide.

15. The process of claim 13, wherein the staining mixture is subjected to a temperature of about 4°C to about 30°C.

16. The process of claim 14, wherein the staining mixture is subjected to a temperature of about 30°C to about 39°C.

17. The process of claim 14, wherein the staining mixture is subjected to a temperature of about 40°C to about 50°C.

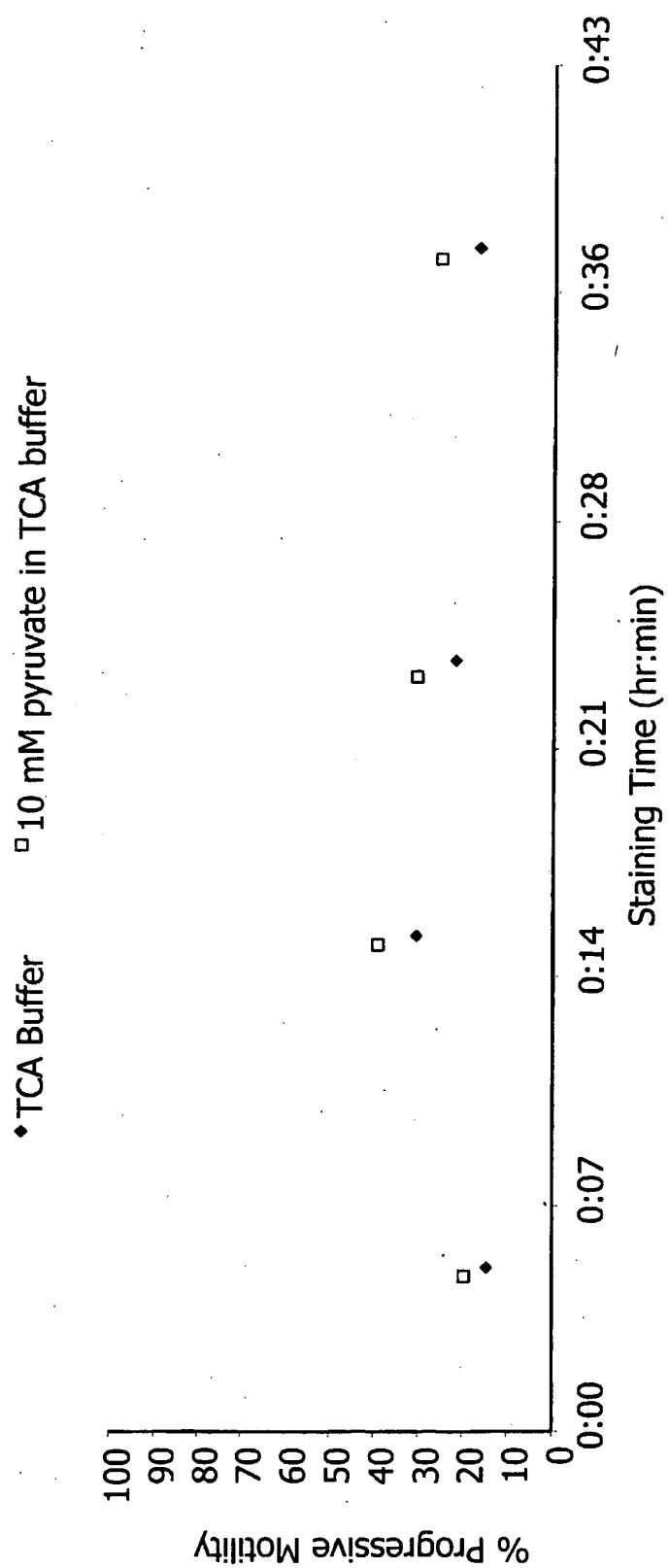
18. The process of claim 13, wherein the composition comprises pyruvate at a concentration selected from the group consisting of about 2.5mM, about 10mM, about 15mM, about 25mM, and about 50mM.

19. The process of claim 13, wherein the composition comprises vitamin K at a concentration selected from the group consisting of about  $10\mu\text{M}$ , about  $50\mu\text{M}$ , about  $75\mu\text{M}$ , and about  $100\mu\text{M}$ .

20. The process of claim 13, wherein the composition comprises lipoic acid at a concentration selected from the group consisting of about  $0.1\text{mM}$ , about  $0.5\text{mM}$ , about  $0.75\text{mM}$ , about  $1.0\text{mM}$ , and about  $1.5\text{mM}$ .

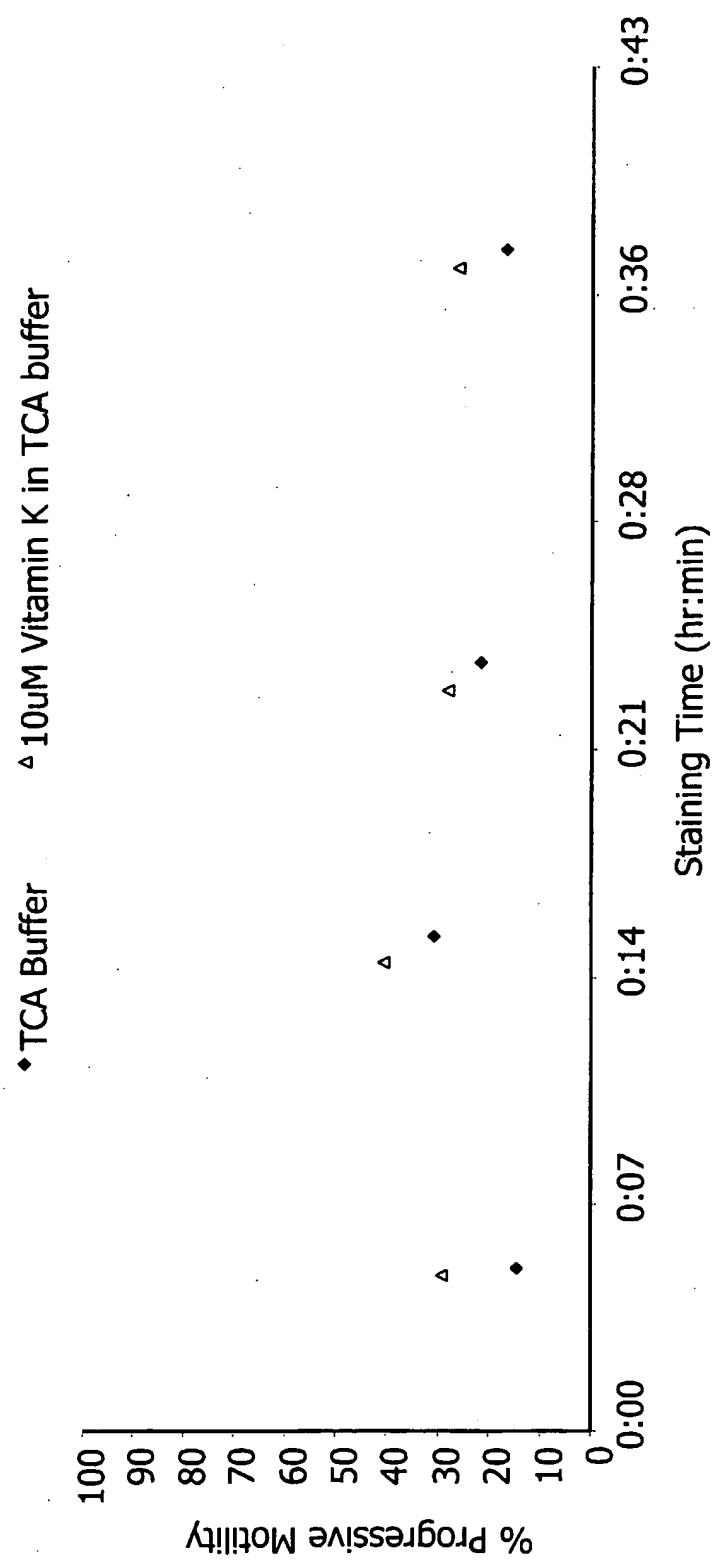
1/20

FIG. 1



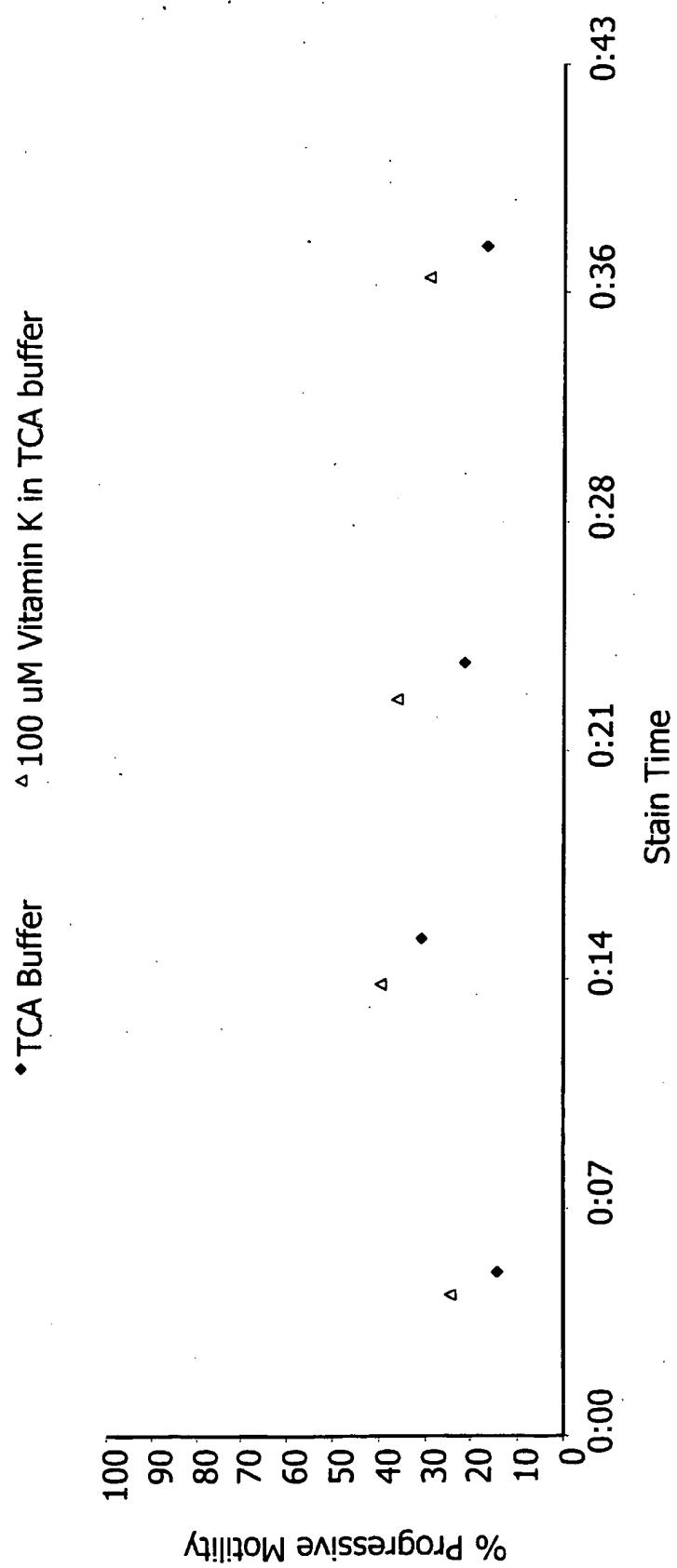
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FIG. 2



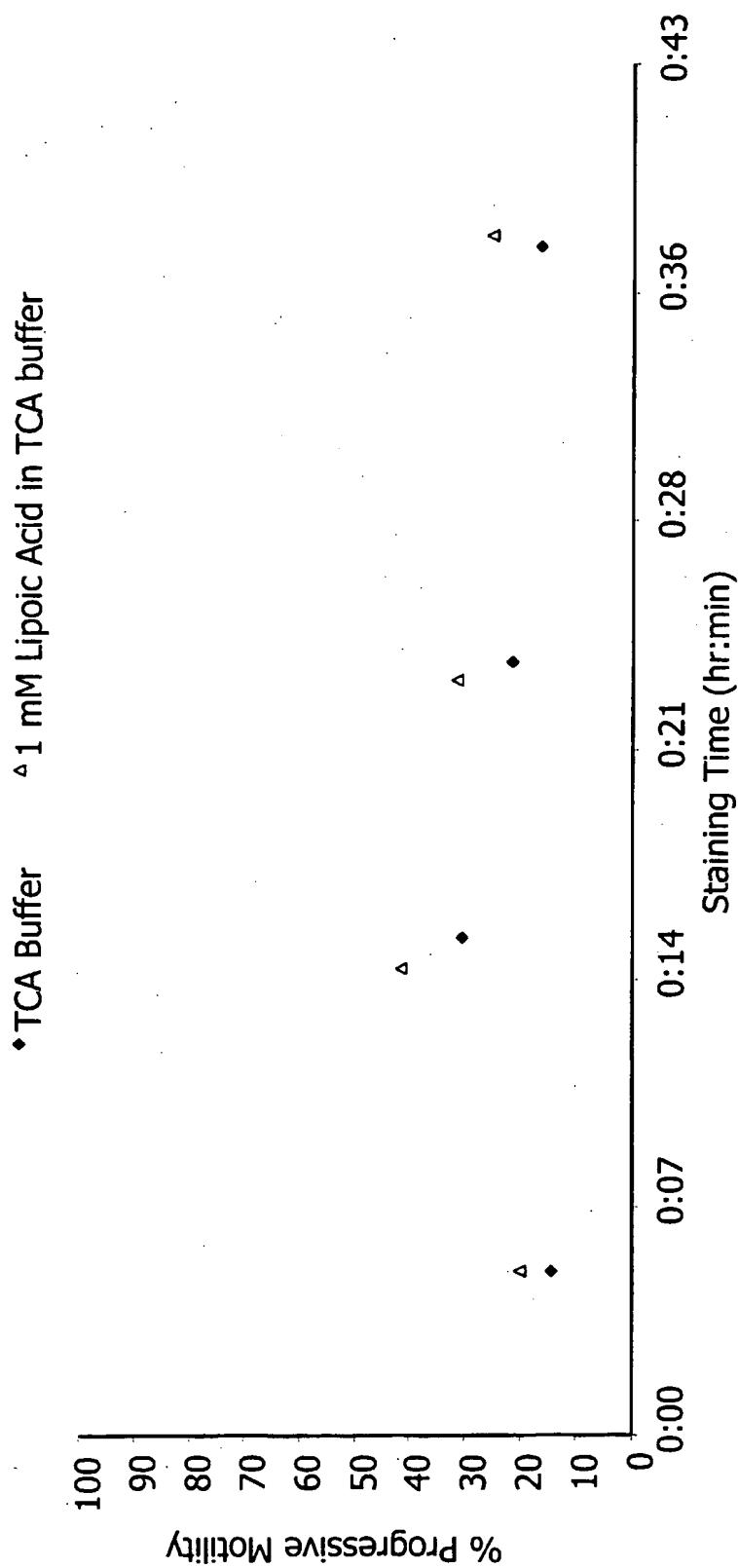
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FIG. 3



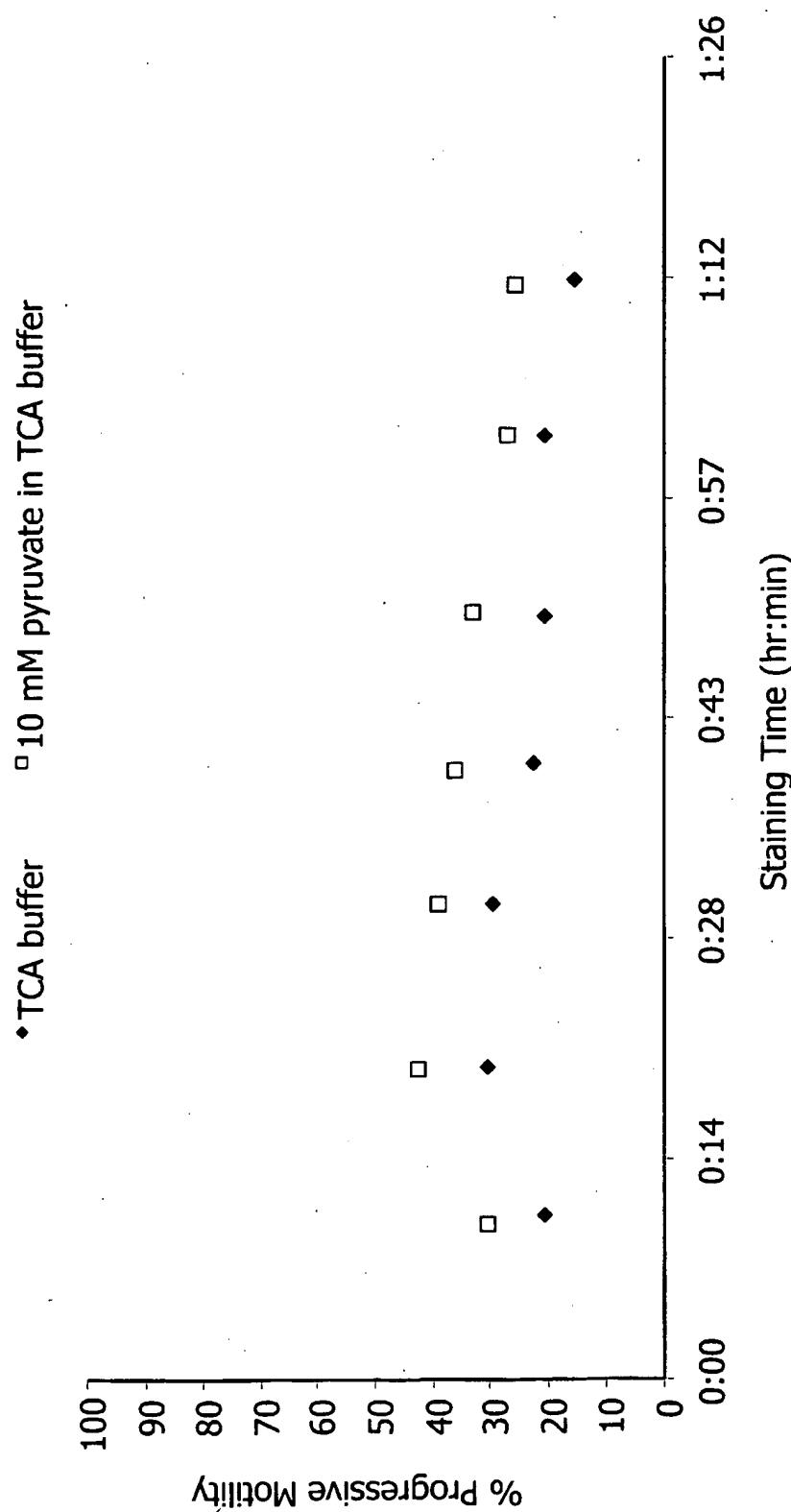
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FIG. 4



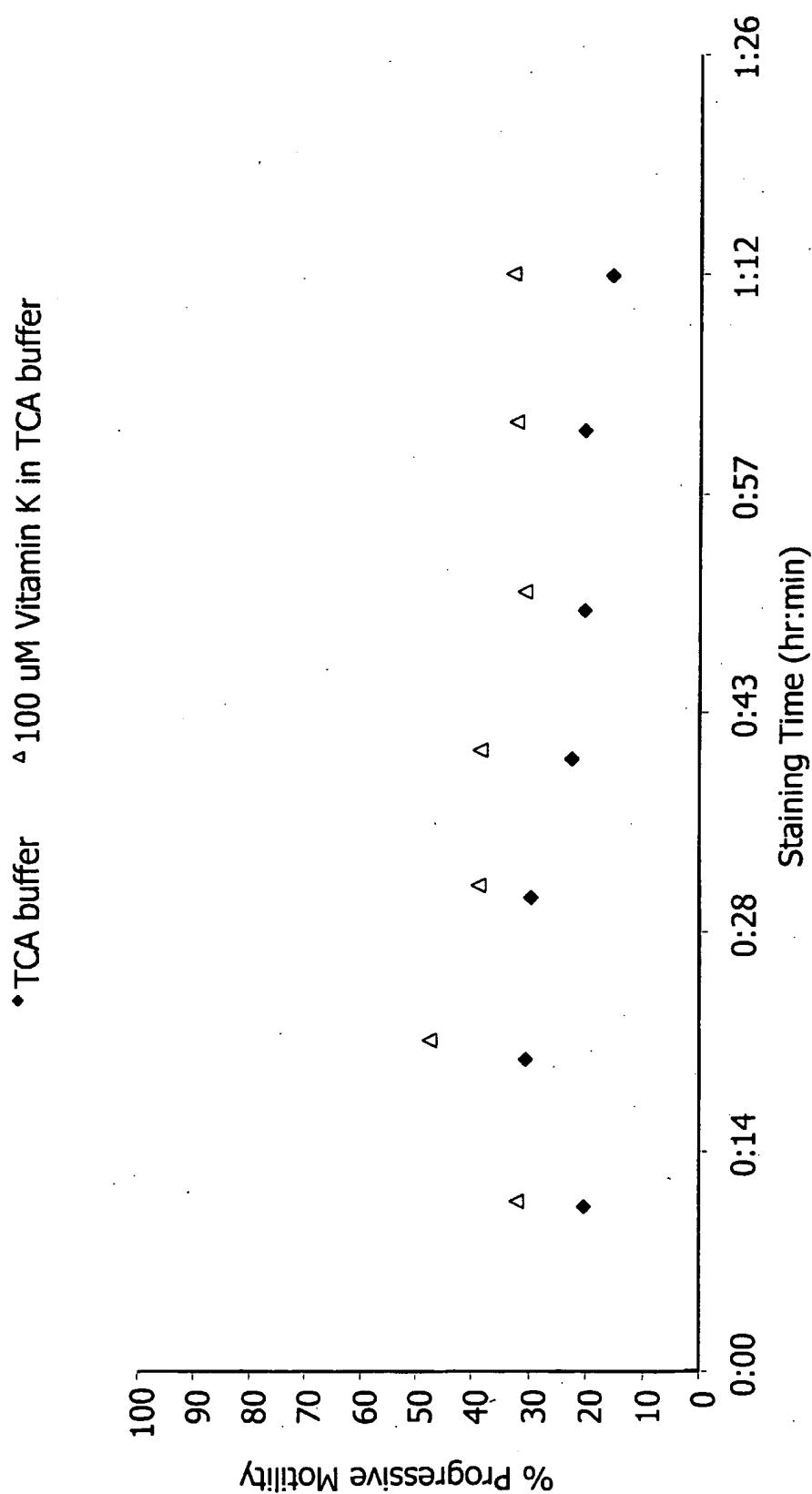
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FIG. 5



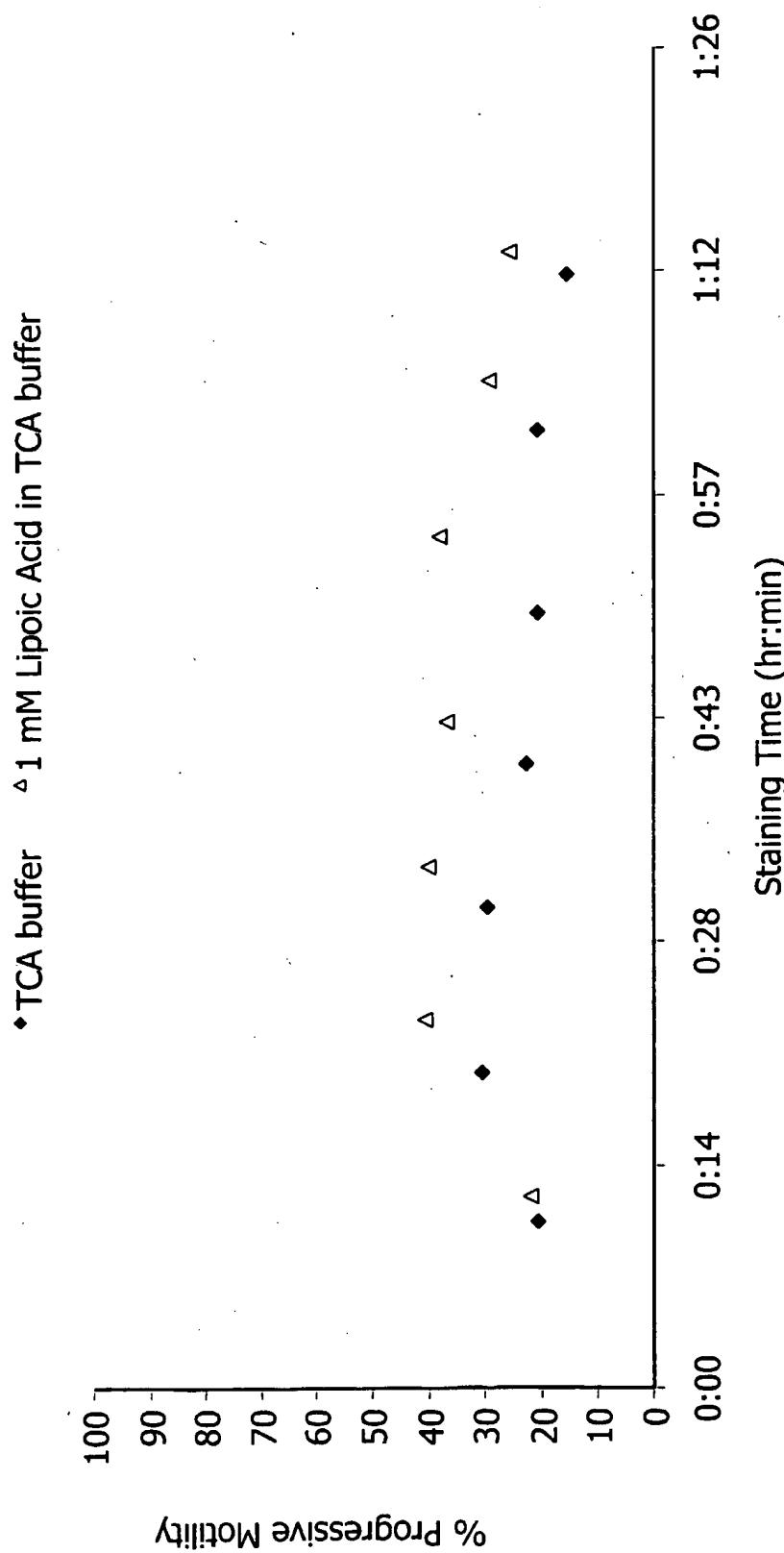
6/20

FIG. 6



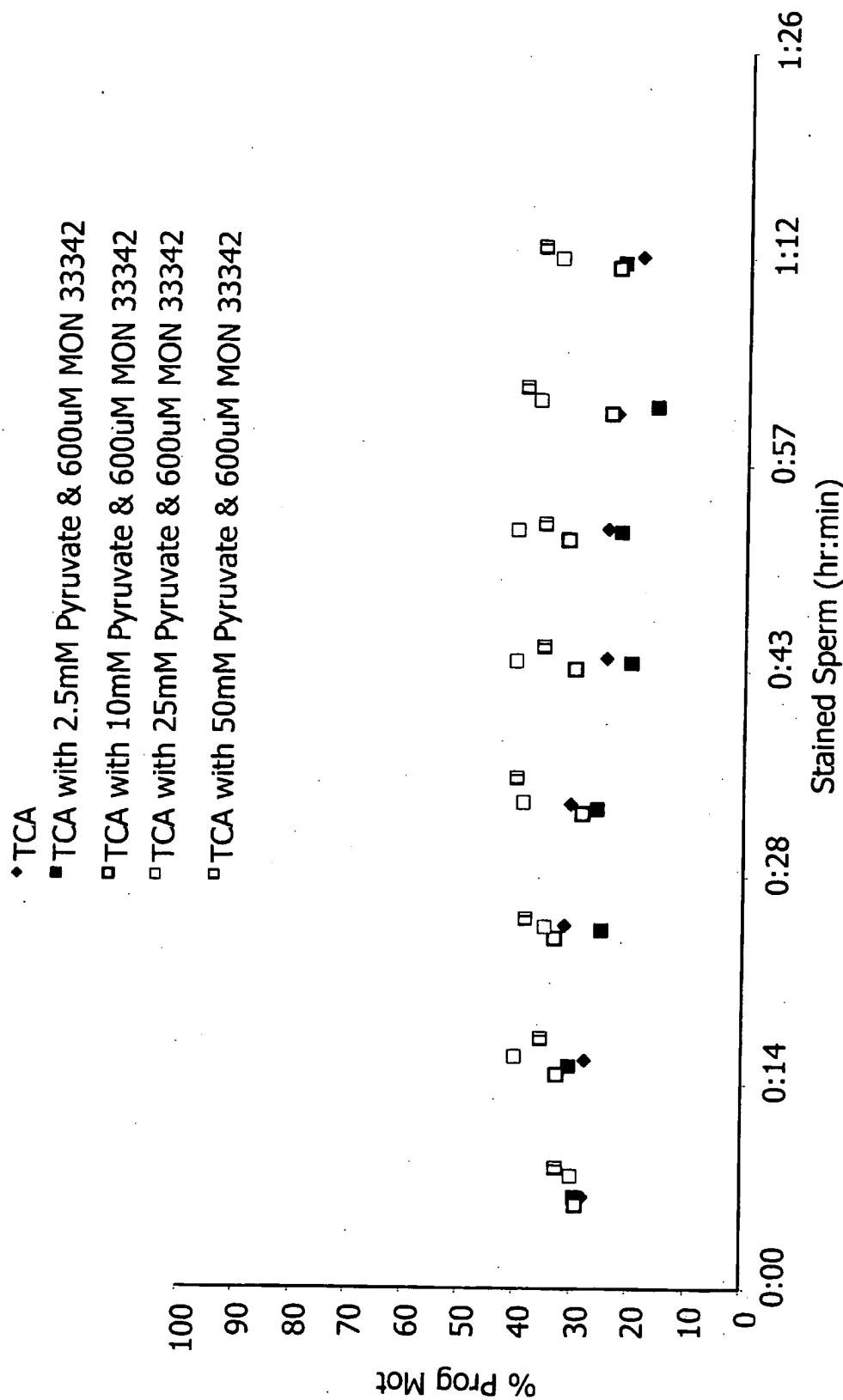
7/20

FIG. 7



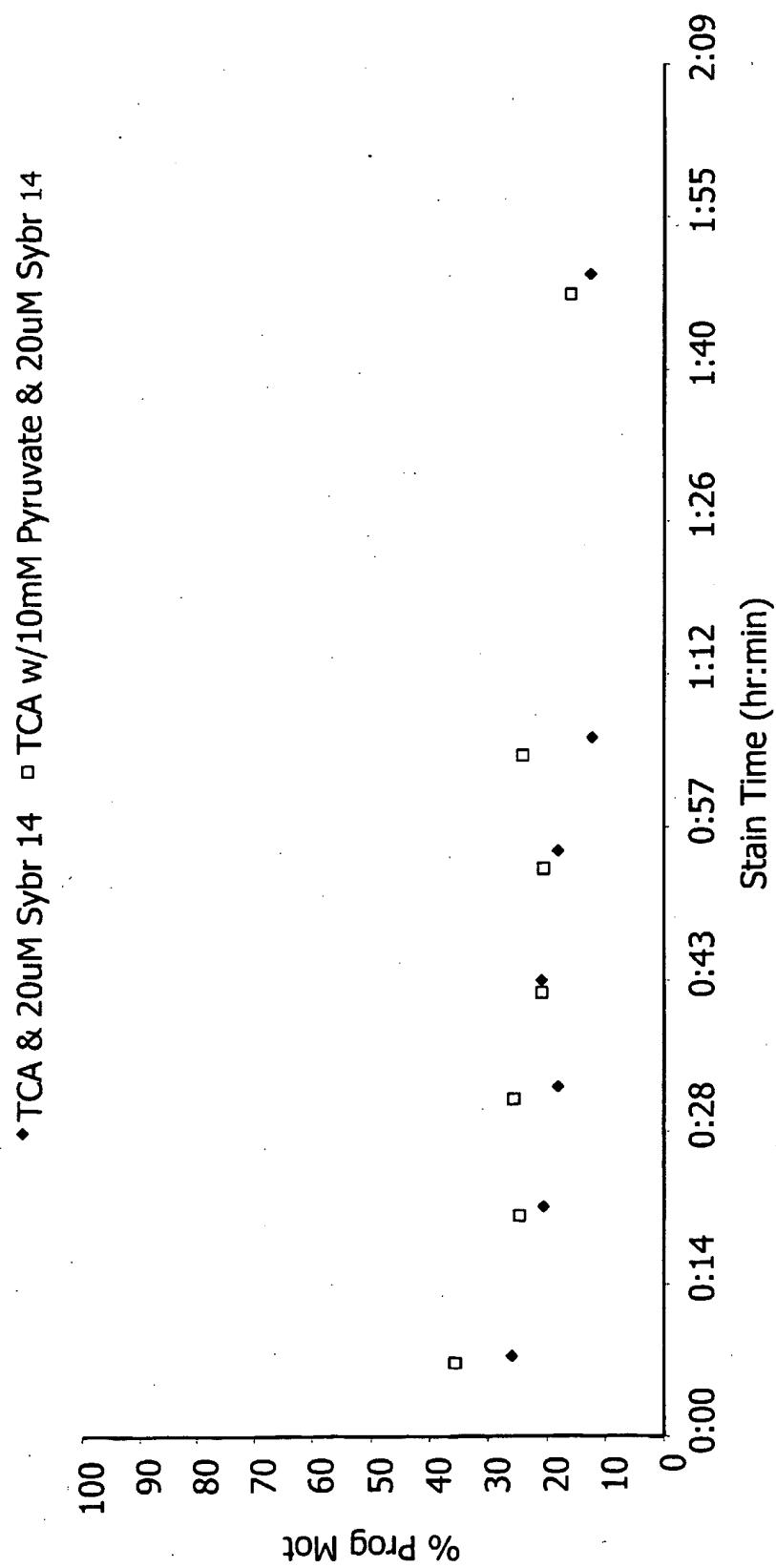
8/20

FIG. 8



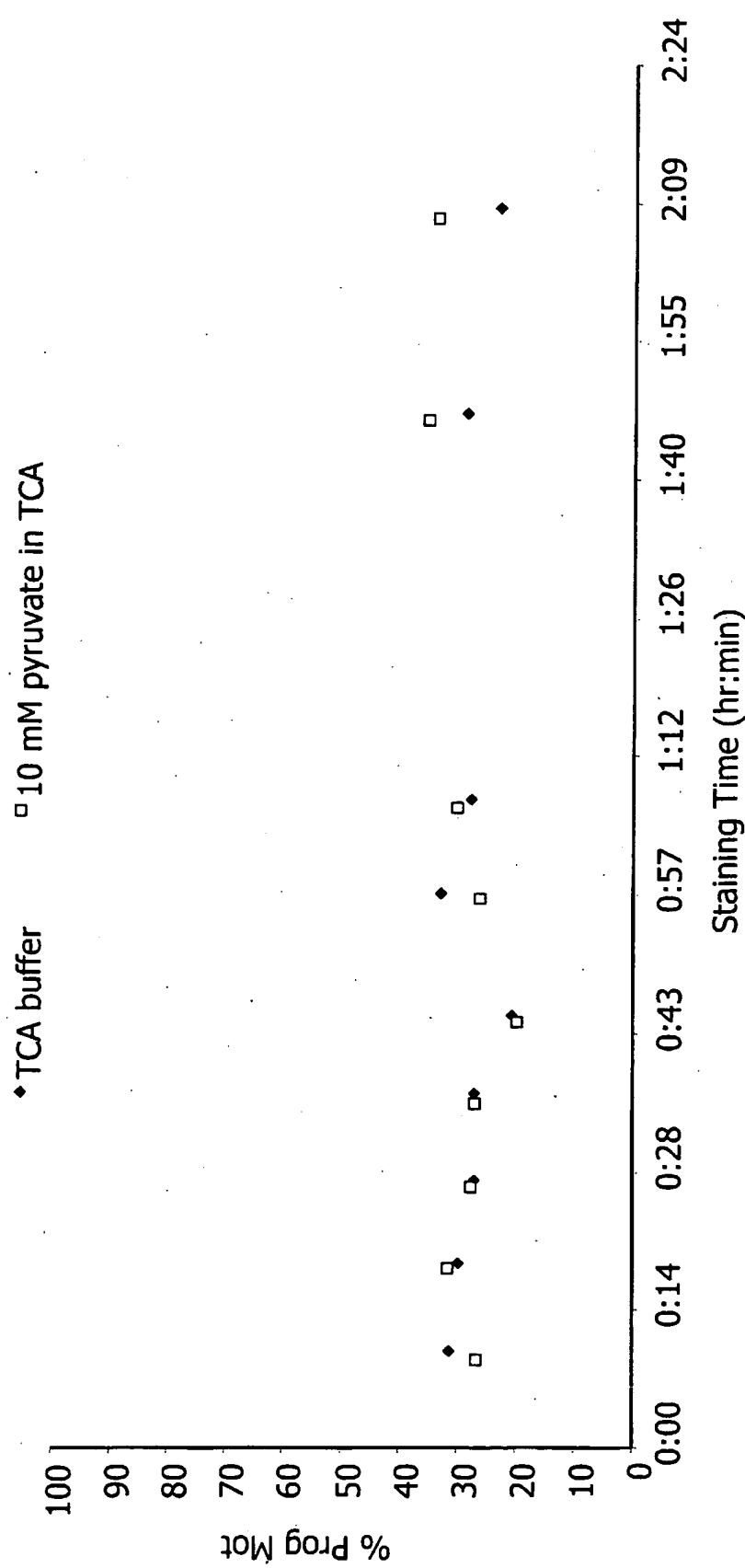
9/20

FIG. 9



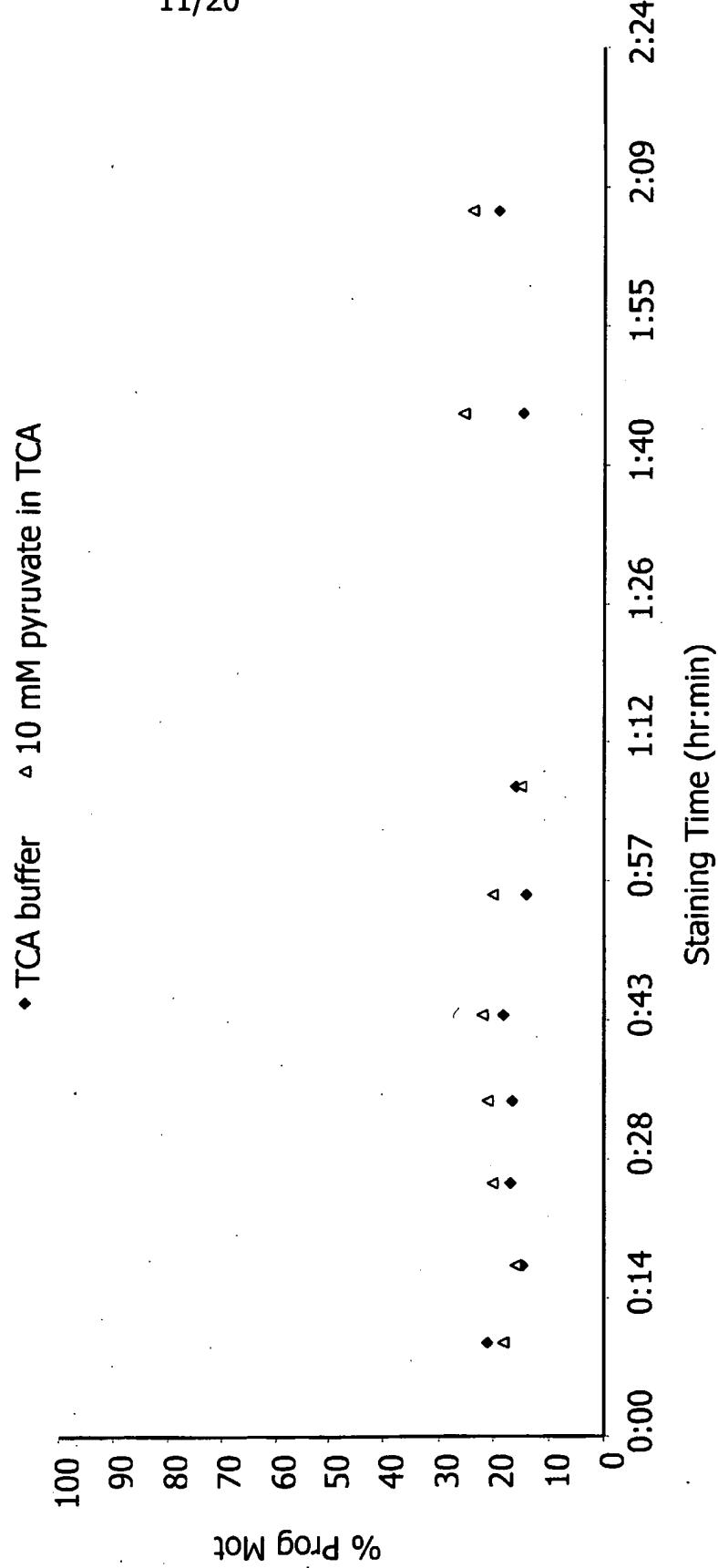
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FIG. 10



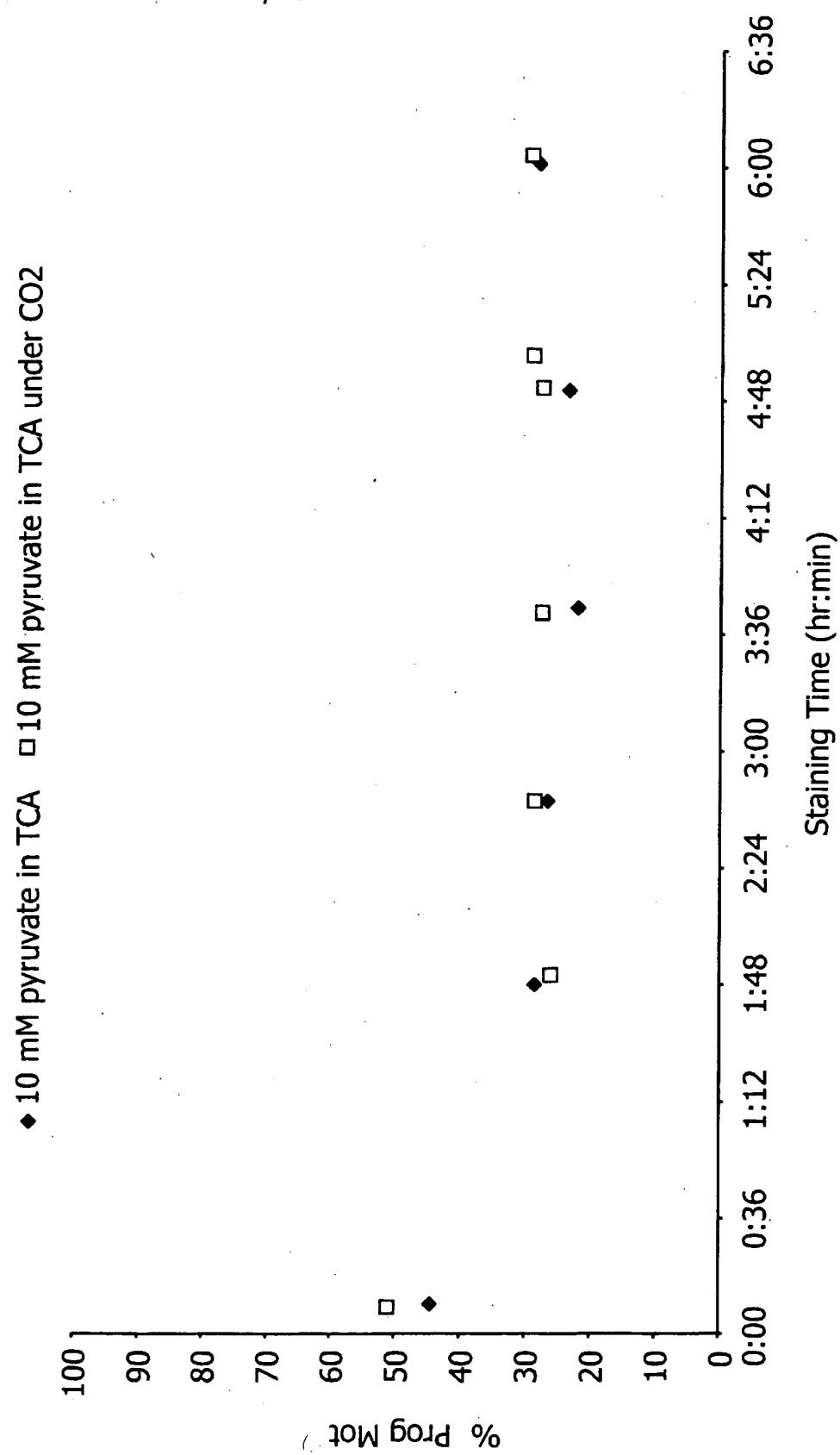
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FIG. 11



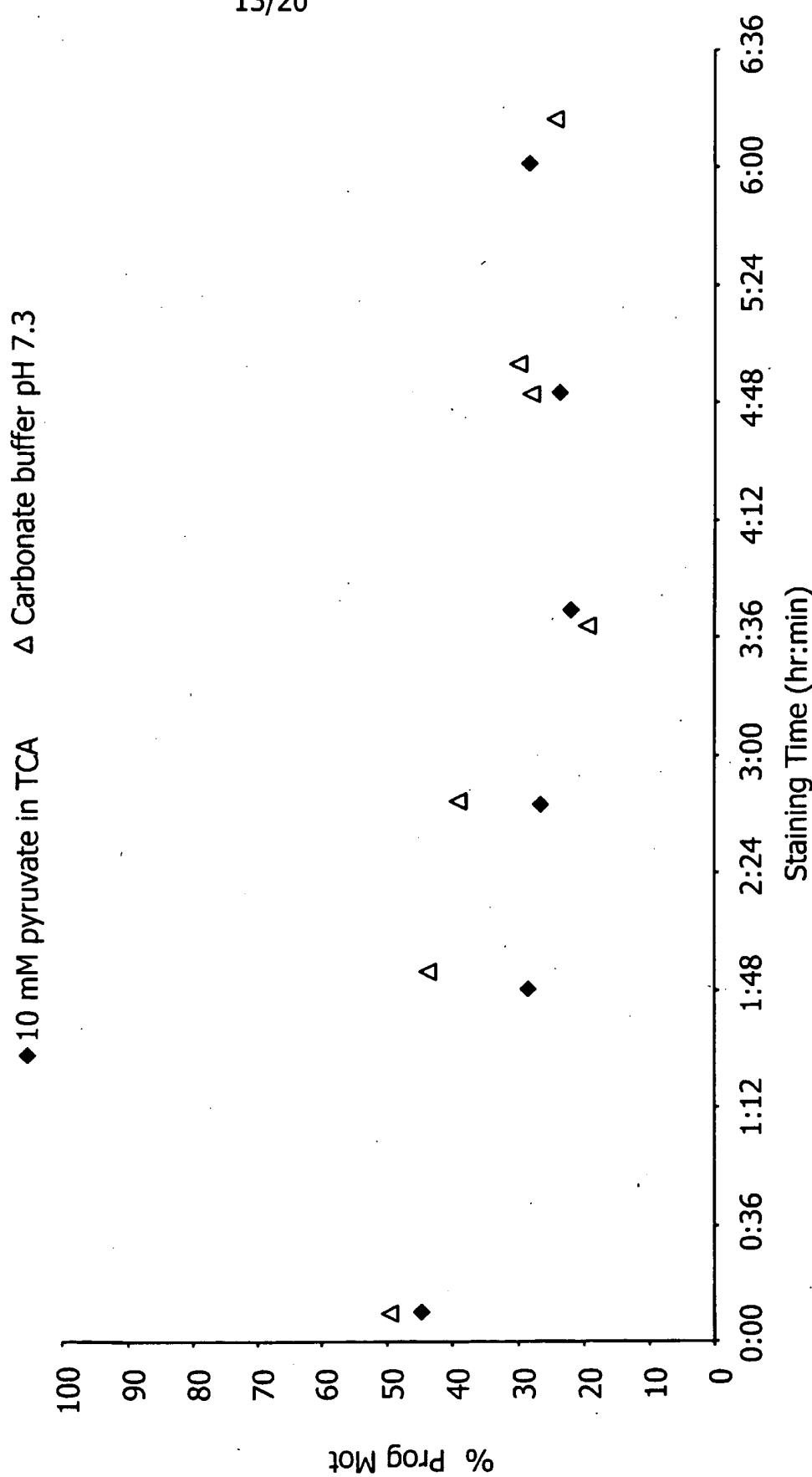
12/20

FIG. 12



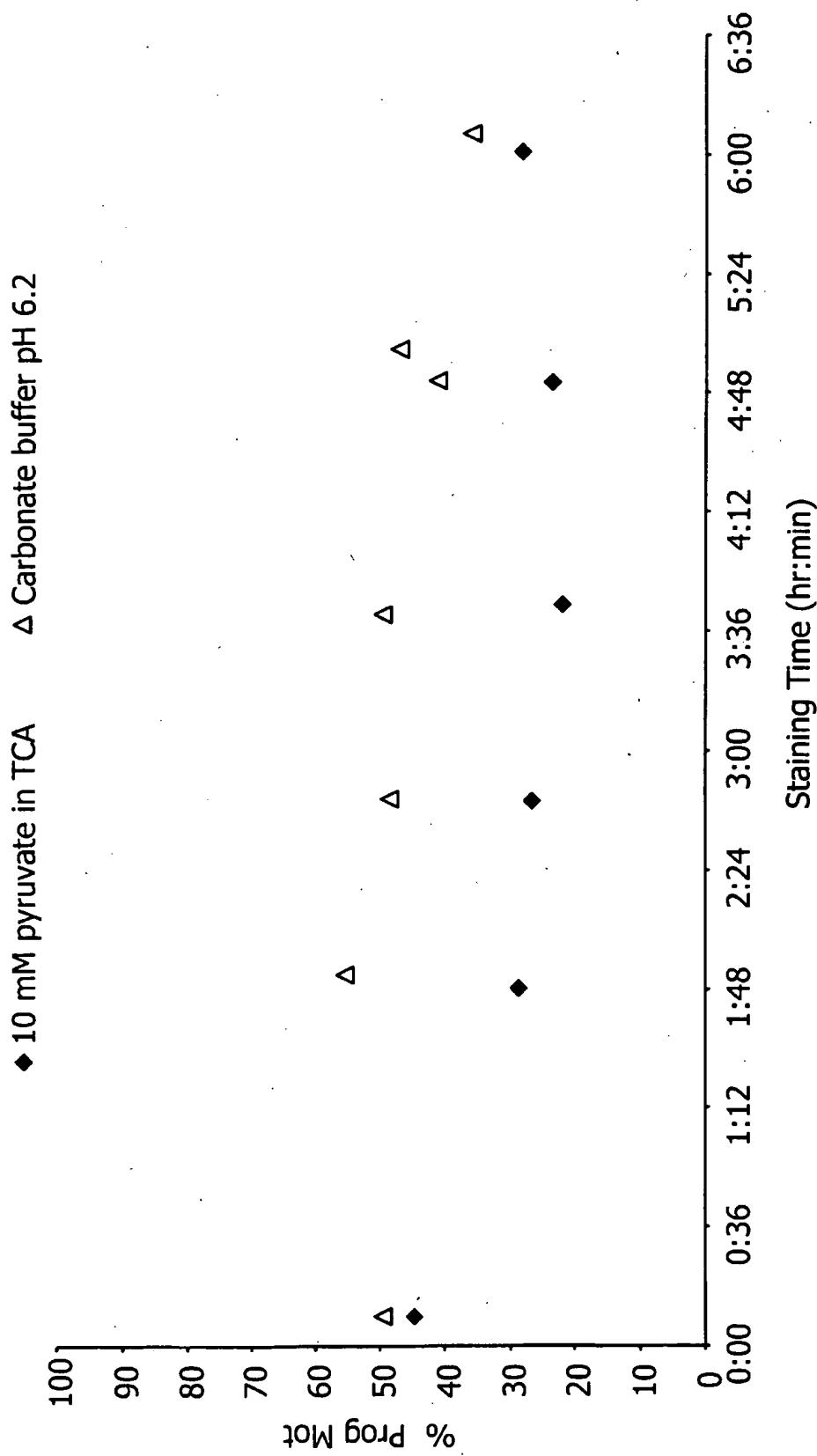
13/20

FIG. 13



14/20

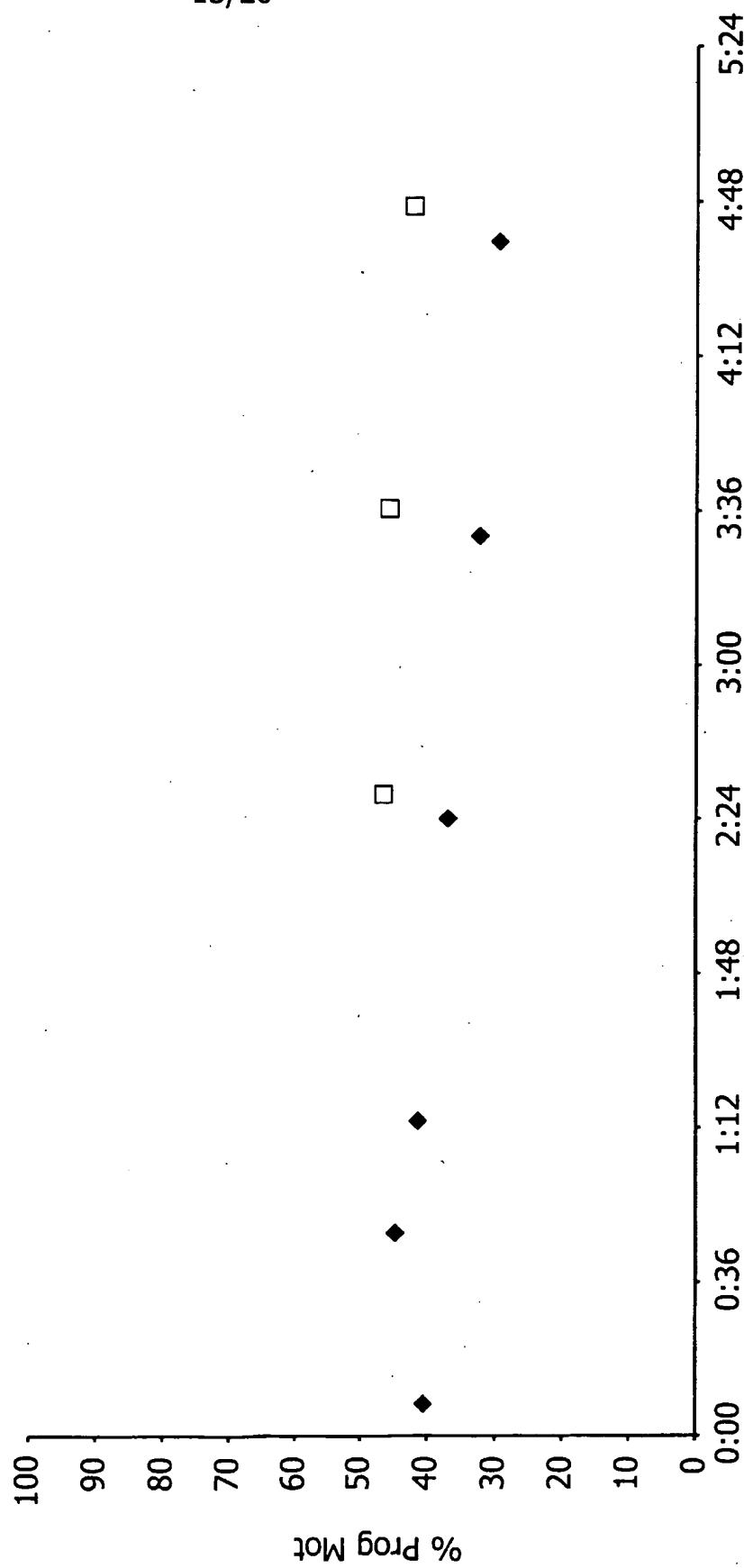
FIG. 14



15/20

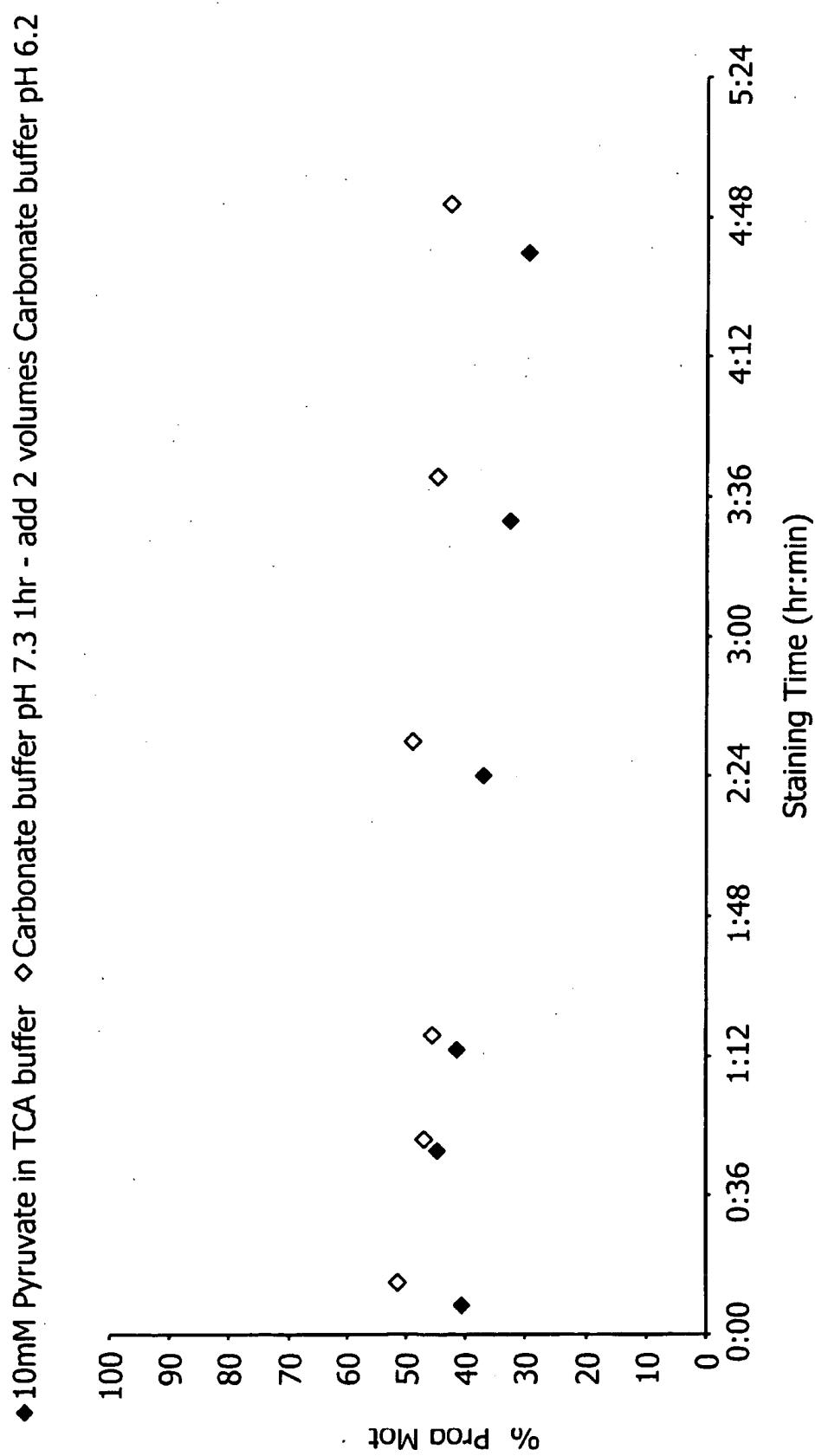
FIG. 15

◆ 10mM Pyruvate in TCA buffer □ 10 mM pyruvate in TCA for 1 hour add 2 volumes carbonate buffer pH 6.2



16/20

FIG. 16



17/20

FIG. 17

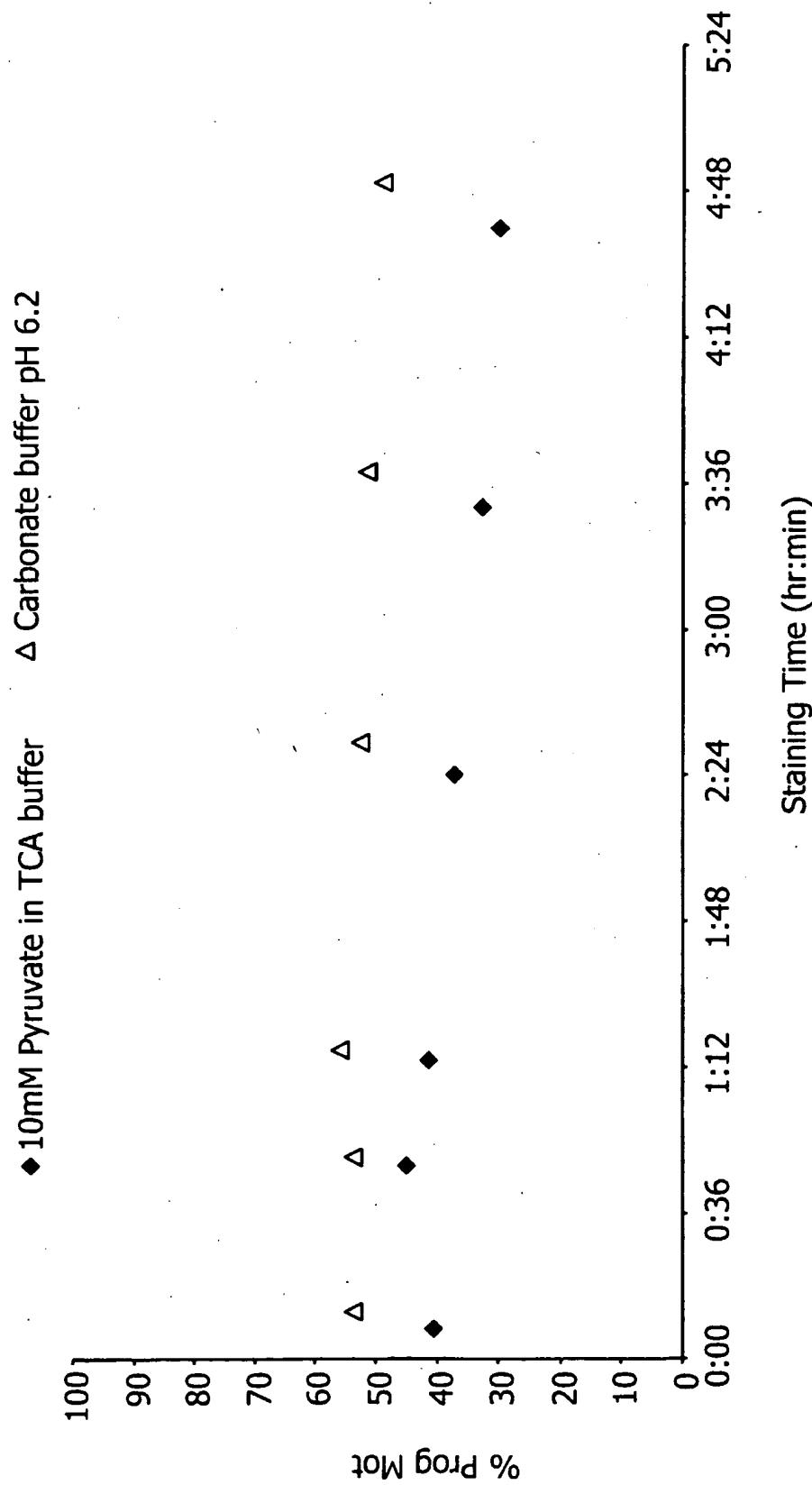


FIG. 18

18/20

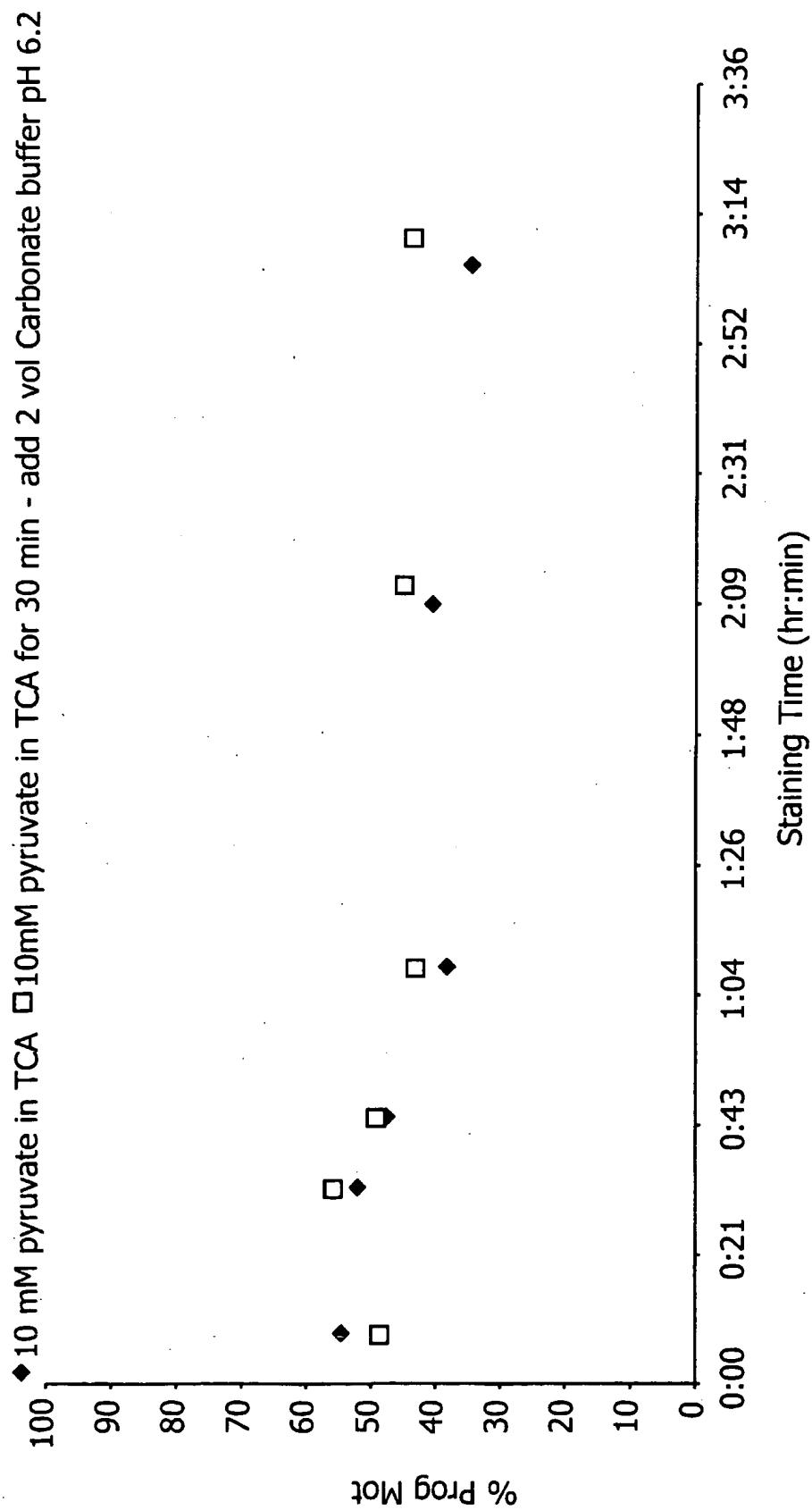


FIG. 19

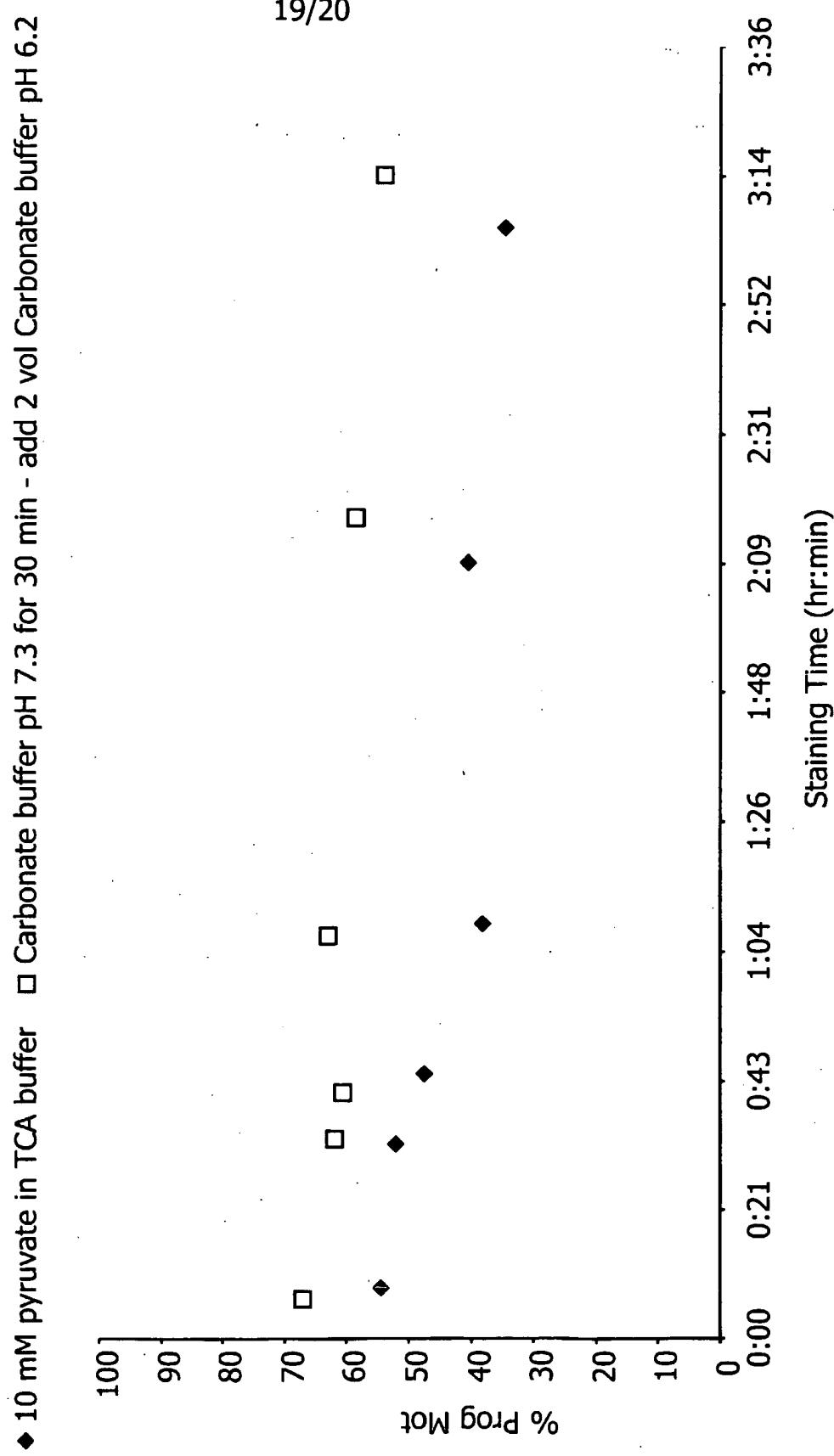
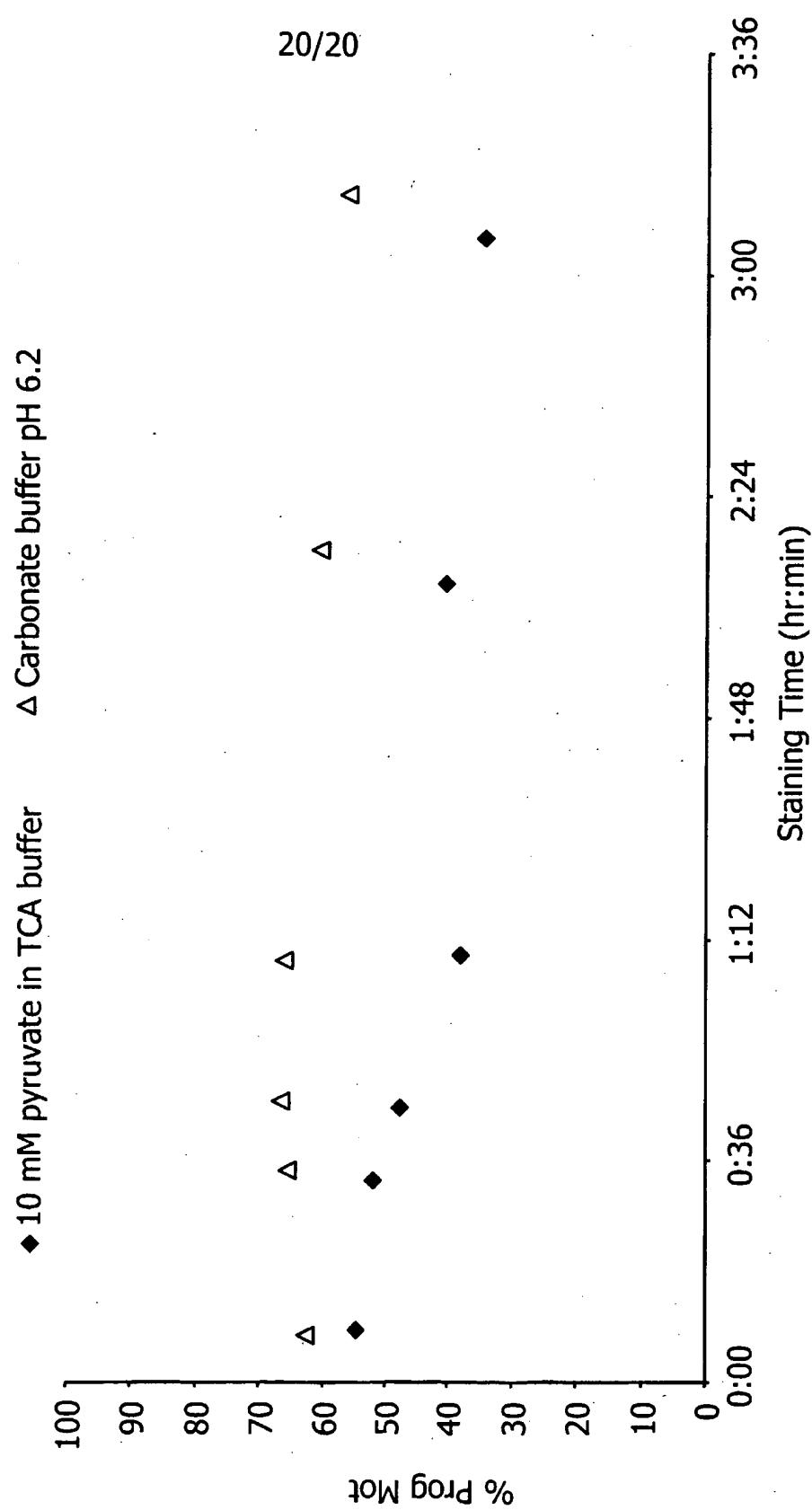


FIG. 20



# INTERNATIONAL SEARCH REPORT

International Application No.  
PCT/US2005/010598

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 7 G01N33/50 C12N5/06

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 7 G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the International search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, PAJ, BIOSIS, MEDLINE, EMBASE, SCISEARCH, CHEM ABS Data, BIOTECHNOLOGY ABS

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 02/41906 A (PHARMACIA CORPORATION; DIDION, BRADLEY; BASKIN, JAMES; WOODARD, SCOTT) 30 May 2002 (2002-05-30) cited in the application the whole document ----- A BRUEMMER J E ET AL: "Effect of pyruvate on the function of stallion spermatozoa stored for up to 48 hours" JOURNAL OF ANIMAL SCIENCE, vol. 80, no. 1, January 2002 (2002-01), pages 12-18, XP001206850 ISSN: 0021-8812 the whole document ----- -/-	1-20

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

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27/06/2005

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## INTERNATIONAL SEARCH REPORT

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	<p>BAUMBER JULIE ET AL: "The effect of reactive oxygen species on equine sperm motility, viability, acrosomal integrity, mitochondrial membrane potential, and membrane lipid peroxidation"  JOURNAL OF ANDROLOGY,  vol. 21, no. 6, November 2000 (2000-11),  pages 895-902, XP009049259  ISSN: 0196-3635  abstract  page 896, left-hand column, paragraph 2.  experiments 2,3  discussion</p> <p>-----</p> <p>DENNISTON, D. J. ET AL: "Effect of antioxidants on the motility and viability of cooled stallion spermatozoa"  JOURNAL OF REPRODUCTION AND FERTILITY,  SUPPLEMENT , 56(EQUINE REPRODUCTION VII),  121-126 CODEN: JRFSAR; ISSN: 0449-3087,  2001, XP009049266  the whole document, especially table 2</p> <p>-----</p> <p>WO 2004/087177 A (MONSANTO TECHNOLOGY LLC;  ANZAR, MUHAMMAD; LUDWIG, CINDY, L; GRAHAM,  JE) 14 October 2004 (2004-10-14)  the whole document, especially paragraphs  58, 61, examples 20-24, figures 20-26 and  claims 1, 29-38</p> <p>-----</p> <p>WO 2004/088283 A (MONSANTO TECHNOLOGY LLC;  DURACK, GARY; HATCHER, JEREMY, T;  WESTFALL, L) 14 October 2004 (2004-10-14)  page 25, line 13 - line 18  page 26, line 39 - page 27, line 15  page 29, line 27 - line 34  example 4</p> <p>-----</p>	
P, X		1-20
P, X		1-20

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No  
PCT/US2005/010598

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		WO 2004087177 A1		14-10-2004

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